Metabolism and Infection in the *Stagonospora nodorum*-Wheat Pathosystem

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I declare that this thesis is my own account of my own work and contains as its main content work which has not been previously submitted for any degree at any tertiary institution.

……

Ormonde Dominick Creagh Waters.

Ode to an Ectopic Fungal Mutant (Pmk1-61)

Thy hyphae fair didst bloom upon my plate
Of medium minimal, yet enough to grow.
And with selective fungicides to ensure
Lest non-transformants would contaminate.
In Stygian darkness, but near-UV also
I nourished you and waited you to spoor.
A picture portrait I did make of you,
Your handsome colours did my eye delight
And I did hope that you might be the one!
An homologous recombinant mutant – Oh so true
On you an Honours chapter I would write
And you a thesis cover would become.
Alas! By PCR you proved ectopic
And now you moulder in a bin necrotic.

Ormonde Waters 2007

ABSTRACT

Stagonospora nodorum is a necrotrophic fungal pathogen, and the causal agent of stagonospora nodorum blotch of wheat. Despite the economic importance of this disease, the molecular basis of the pathosystem is poorly understood. The aim of this study was to investigate the interaction between metabolism and infection in this pathosystem, with particular reference to the metabolism of mannitol.

In common with many fungi, the main metabolite produced by *S. nodorum* is the acyclic hexitol mannitol. Among the previously suggested roles for this compound is a role in pathogenicity. The metabolism of mannitol has been hypothesised as occurring in a cycle involving the enzymes mannitol 2-dehydrogenase (*Mdh1*) and mannitol 1-phosphate 5-dehydrogenase (*Mpd1*). A strain was created harbouring disruption constructs for both of these genes. The double mutant was unable to synthesise or catabolise mannitol, and was unable to sporulate. Addition of exogenous mannitol completely restored *in vitro* sporulation, and partially restored *in planta* sporulation. This demonstrated an essential role for mannitol in asexual sporulation. This is the first demonstrated role for this compound.

A ^{13}C NMR study of the wild type strain, the *mdh1* and *mpd1* single mutants, and *mpd1mdh1* double mutant was undertaken to investigate carbon utilisation and cycling. Disruption of *Mpd1* significantly altered the metabolite profile with the *mpd1* mutants producing trehalose and glycerol in place of mannitol. Labelling patterns in the double mutant showed that scrambling of label can be explained by the

triosephosphate isomerase triangle and pentose phosphate pathway. This suggests the contribution of mannitol to label scrambling has been overstated in previous studies.

The evidence did not support the metabolism of mannitol in *S. nodorum* as occurring in a cycle, but rather as two separate pathways.

A GC-MS analysis of diseased and non-diseased tissue from infected leaves, compared to non-infected and mock-inoculated leaves, could not detect any metabolites associated with a systemic host reaction to pathogen attack.

ACKNOWLEDGEMENTS

When the opportunity arose to undertake PhD studies with Prof. Richard Oliver and Dr. Peter Solomon, it required no lengthy consideration. Having completed my Honours degree with these same supervisors, and being familiar with the ACNFP, I knew that I was in good hands and that in addition to undertaking some seriously good science, there would be no shortage of fun along the way. In this I have not been disappointed and I am very grateful to my supervisors for being willing to take on a rather more mature (in years) student than average. I suspect that the social and cultural baggage I have accumulated along the way has not always been conducive to the best scientific method and approach, and they have borne my idiosyncrasies well. There has been a lot of laughter along the way and that is never a bad thing! My candidature has been rather complicated by the arrival of a third child, the lengthy hospitalisation my mother, and two root canals. In between these there have been some exciting moments in the lab, not least of which was the creation of the elusive double mutant *mpd1mdh1* strain. I am grateful to Yair Shachar-Hill of Michigan State University for introducing me to the world of NMR spectroscopy and to Rob Trengove and the Murdoch Separation Science Laboratory for assistance with aspects of the GC-MS part of the study. A big thank you is due to all members of the ACNFP for their camaraderie and support. In particular our wonderful RAs, especially Kasia Rybak, and my fellow stagonaut PhD students (some now post-docs) Kar-Chun Tan, Rohan Lowe, Simon Ip Cho, James Hane, Joel Gummer and Christian Krill. I have been very fortunate to have been funded by the Grains Research and Development Corporation and their support is very sincerely appreciated.

I would like to dedicate this PhD to my family who have offered ongoing love and support. In particular my parents Ormonde and Mina, and my parents-in-law Neville (deceased) and Daphne Harris, and my three great wonders – Clare, Oscar and Fintan (who have been promised a camping trip once I submit – apologies for the delay). Most importantly I dedicate this to Suzanne who knows me better than myself, puts up with a lot and gives so much, and is the sun, moon, stars and music of my life…agus do Íosa mhín, mo Thiarna, do chum ghlóire Dé agus d'onóir Éireann 's An Astráil.

ABBREVIATIONS

1-P	1-phosphate
6-P	6-phosphate
ACNFP	Australian Centre for Necrotrophic Fungal Pathogens
b	base(s)
BCA	bicinchoninic acid
BLAST	Basic Local Alignment Search Tool
bp	nucleotide base pair(s)
BSA	bovine serum albumin
cDNA	complementary DNA
CFE	cell-free extract
cm	centimetre(s)
cv.	cultivar
CzV8CS	Czapek Dox V8 juice complete supplement
DLA	detached leaf assay
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate(s)
dpi	days post inoculation/days post infection
EC	Enzyme Commission
EDTA	ethylenediaminetetra-acetic acid, disodium salt pH 8.0
EST	expressed sequence tag
f. sp.	*forma(e) species*
FT	Fourier transform
g	gram(s)

g	gravity
GC-MS	Gas Chromatography-Mass Spectrometry
gDNA	genomic DNA
GFP	Green Fluorescent Protein
GPS™-M	Genome Priming System - Mutagenesis
h	hour(s)
HSD	honestly significant difference
HST	host-specific toxin
kb	kilobase(s)
kPa	kiloPascal(s)
kV	kilovolt(s)
L	litre(s)
µg	microgram(s)
µL	microlitre(s)
µM	microMolar
µm	micron(s)
M	Molar
MAP kinase	mitogen-activated protein kinase
Mb	Megabase(s)
Mdh1/Mdh1	mannitol 2-dehydrogenase (gene/protein)
mg	milligram(s)
min	minute(s)
mL	millilitre(s)
MM	minimal medium
MM-C	minimal medium minus carbon

mM	milliMolar
mm	millimetre(s)
mol	mole(s)
MPa	Mega Pascal(s)
Mpd1/Mpd1	mannitol-1-phosphate 5-dehydrogenase (gene/protein)
NA	natural abundance
NAD^+	nicotinamide adenine dinucleotide (oxidised)
NADH	nicotinamide adenine dinucleotide (reduced)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidised)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
ng	nanogram(s)
nm	nanometre(s)
NMR	nuclear magnetic resonance
PCA	Principal Components Analysis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEP	phosphoenolpyruvate
pers. comm.	personal communication
pH	potential of hydrogen
P_i	inorganic phosphate
pl.	plural
ppm	parts per million
QTL	quantitative trait locus/loci
qPCR	quantitative polymerase chain reaction
rcf	relative centrifugal force

RNA	ribonucleic acid
RNAase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
SDBS	Spectral Database for Organic Compounds
SDS	sodium dodecylsulphate
SE	standard error
sec	second(s)
sing.	singular
SNB	stagonospora nodorum blotch
sp.	species (sing.)
spp.	species (pl.)
subsp.	subspecies
syn.	synonym
TCA	tricarboxylic acid
TMS	trimethylsilyl
Tween 20	polyoxyethylenesorbitan monolaurate
U	unit(s)
UV	ultraviolet
V	volt(s)
v/v	volume per volume
WT	wild type
w/v	weight per volume

TABLE OF CONTENTS

LIST OF TABLES

Continued on the following page

LIST OF TABLES (contd)

LIST OF FIGURES

Continued on the following page

LIST OF FIGURES (contd)

Continued on the following page

LIST OF FIGURES (contd)

Continued on the following page

LIST OF FIGURES (contd)

Continued on the following page

LIST OF FIGURES (contd)

PAPERS PUBLISHED FROM THIS STUDY

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*As equal first author. My contribution to this paper included the creation and characterisation of the *mdh1* mutant and the *mpd1mdh1* double mutant and the discovery that the double mutant was unable to undergo asexual sporulation without the addition of exogenous mannitol.

A paper reporting the results of the ^{13}C-NMR study conducted here is in preparation.

CHAPTER 1: METABOLISM AND INFECTION

1.1 The Pathosystem Concept

A pathosystem is defined by the phenomenon of parasitism i.e. where one organism, the parasite or pathogen, derives some or all of its energy requirements from a second organism, a living host (Robinson, 1976). Every living organism is potentially a host, but can only actually be defined as such when another organism becomes parasitic upon it. A pathosystem can therefore be defined as the biological relationship which exists between a single pathogen and a single host.

In the case of plant pathosystems, the pathogens may be fungi, mycoplasmas, bacteria, nematodes, oomycetes, viruses, or viroids. Occasionally it may be another plant (March and Watson, 2007), but the definition does not extend to herbivory or to the more mutualistic relationships between plants and mycorrhizal fungi, or insect and mammalian pollination vectors.

The genomes of the participating organisms are the result of ongoing selection pressures. They will continue to be shaped by such pressures, although the nature and intensity of the pressures may alter over time. One particular selection pressure which is applicable to a pathosystem is the asymmetric “evolutionary arms race” whereby the host species are under selection pressure to favour the progeny of those individuals with better mechanisms of resistance to the pathogen, while the pathogen species will be under selection pressure to favour the progeny of those individuals with better mechanisms of overcoming host resistance (Dawkins and Krebs, 1979). These selection pressures can also be influenced by the nature of the host-pathogen

relationship which has been traditionally categorised as biotrophic or necrotrophic (reviewed in Oliver and Ipcho, 2004).

Biotrophs are typically obligate pathogens with a narrow host range, causing little damage to their host and feeding off living cells via haustoria, engaging in classical gene-for-gene interactions, and with the hypersensitive response being a feature of incompatible interactions (Both and Spanu, 2004). Necrotrophs are characterised by being non-obligate with a broad host range, production of cell-wall degrading enzymes and toxins, feeding off dead or dying host cells, and with host resistance being polygenic (Oliver and Ipcho, 2004). Secreted toxins include host-specific toxins (HSTs) which are important determinants of host range and may be proteinaceous or low molecular weight compounds (Scheffer, 1983; Sarpeleh *et al.*, 2007). These include ToxA, initially characterised in the *Pyrenophora tritici-repentis*-wheat pathosystem (Tuori *et al.*, 1995; Ciuffetti *et al.*, 1997), and subsequently shown to have most likely originated in *Stagonospora nodorum*, providing the most convincing evidence to date for eukaryotic interspecific virulence gene transfer (Friesen *et al.*, 2006). A special sub-category of necrotrophs is the hemibiotrophs, which commence with an asymptomatic infection and after a latent period switch to a host-cell destructive necrotrophic mode (Oliver and Ipcho, 2004).

The pathosystem which was the focus of this study is that occurring between the host plant wheat (*Triticum aestivum* subsp. *aestivum*) and the necrotrophic fungal pathogen *Stagonospora nodorum*.

1.2 The *Stagonospora nodorum*-Wheat Pathosystem

*1.2.1 The Host (*Triticum aestivum *L.)*

Wheat (*Triticum aestivum* L.) is one of the major crops produced worldwide in terms of the amount of arable land reserved for its production, the volume of production, and the value of the crop, with a forecast record production of 658 million tonnes predicted for 2008/09 (FAO, 2008). In Australia, from 2003/04 to 2005/06 over 50% of all land farmed for crops was accounted for by wheat production (Table 1.1). Wheat accounted for over 60% of total cereal production (Table 1.2), and in 2006/07 wheat had a gross value of AUD 5.1 billion (ABS, 2007; ABS, 2008). The cultivation of wheat in Australia occurs in a wide sub-coastal band known as the wheat-belt, which extends around most of the southern half of the continent (Figure 1.1). The two main types of wheat cultivated are bread wheat (*Triticum aestivum* subsp. *aestivum*) and durum or macaroni wheat (*Triticum turgidum* subsp. *durum*), with the latter comprising 3% of the total crop (ABS, 2006).

*1.2.2 The Pathogen (*Stagonospora nodorum*)*

1.2.2.1 Discovery and nomenclature of the organism

Stagonospora nodorum (Berk.) E. Castell. and Germano (1977) [teleomorph: *Phaeosphaeria* (syn. *Leptosphaeria*) *nodorum* (E. Müll.) Hedj. (1968)] was first noted on wheat by Berkeley in 1845 (Weber, 1922). The anamorph has a number of synonyms including *Depazea nodorum* Berk., *Hendersonia nodorum* (Berk.) Petr.,

Table 1.1: Area set aside for production of all crops, for wheat alone, and wheat as a percentage of the total area cropped in Australia from 2002/03 to 2005/06 (Australian Bureau of Statistics, 2007, 2008).

Year	Total Area Under Crops ('000 ha)	Total Area of Wheat ('000 ha)	Wheat as a Percentage of Total Area Cropped
02/03	23,575	11,170	47.4%
03/04	26,080	13,067	50.1%
04/05	26,742	13,399	50.1%
05/06	24,255	12,703	52.4%

Table 1.2: Area, Production and Gross Value of cereal crops grown in Australia from 2002/03 to 2005/06 (Australian Bureau of Statistics, 2007, 2008).

	Area ('000 ha)				Production ('000 t)				Gross Value ($m)			
	2002/03	**2003/04**	**2004/05**	**2005/06**	**2002/03**	**2003/04**	**2004/05**	**2005/06**	**2002/03**	**2003/04**	**2004/05**	**2005/06**
Barley	3,864	4,477	4,646	4,481	3,865	10,382	7,740	9,641	984	1,750	1,233	na
Grain Sorghum	667	734	755	792	1,465	2,009	2,011	1,999	300	319	270	na
Maize	50	70	72	69	310	*395	420	370	72	*88	81	na
Oats	911	1,089	894	945	957	2,018	1,283	1,723	210	279	172	na
Rice	46	66	51	100	438	553	339	982	153	180	101	na
Wheat	11,170	13,067	13,399	12,703	10,132	26,132	21,905	25,704	2,692	5,636	4,317	5.1 bill.
Lupins	1,025	851	845	853	726	1,180	937	1,357	212	278	193	na
Total	**17,733**	**20,354**	**20,662**	**19,943**	**17,893**	**42,274**	**34,635**	**41,776**	**4,623**	**8,442**	**6,367**	-
Wheat (% of Total)	**62.99**	**64.20**	**64.85**	**63.70**	**56.63**	**61.82**	**63.25**	**61.53**	**58.23**	**66.76**	**67.80**	-

*Estimate has a relative standard error of 10% to less than 25% and should be treated with caution.
na =not available

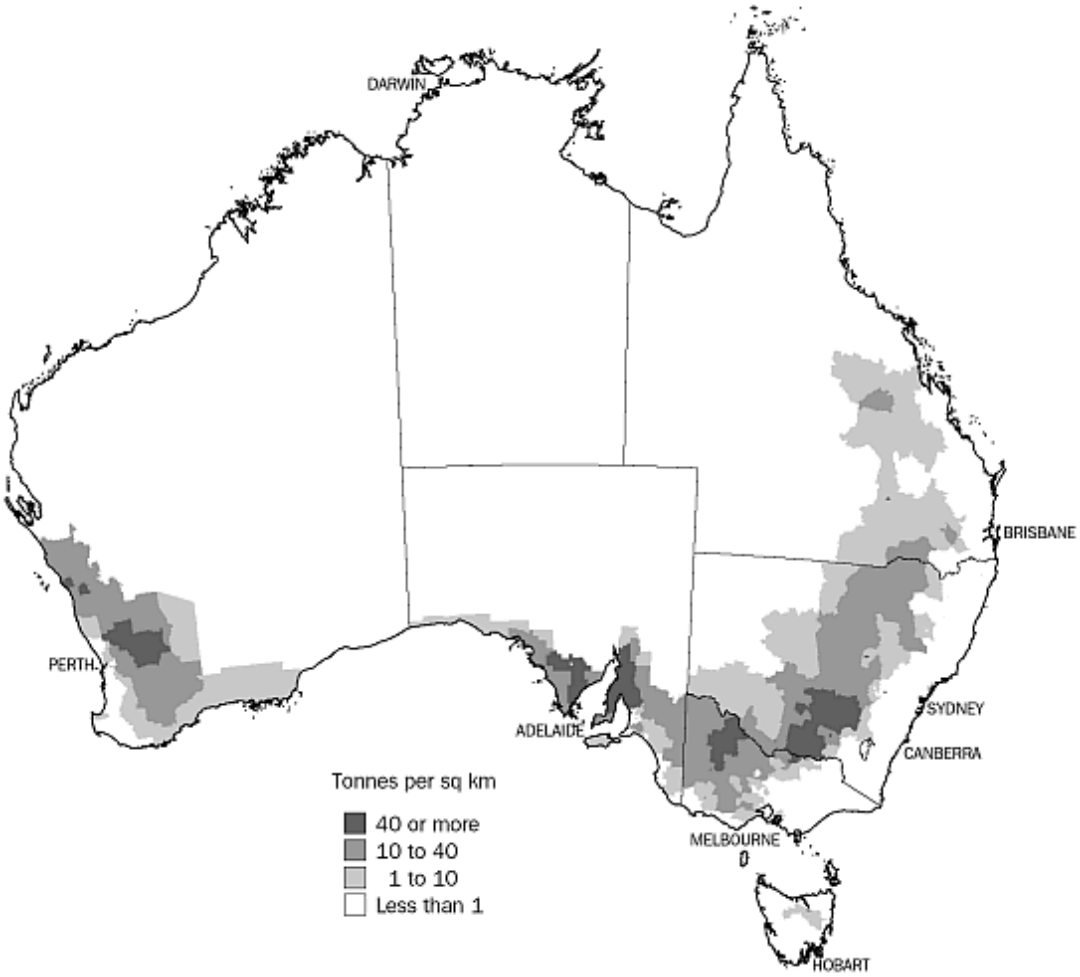

Figure 1.1: Distribution and yield of wheat production in Australia 2000-01.

Source: ABS (2006).

Macrophoma hennebergii (Kühn) Berl. & Vogl., *Phoma hennebergii* (Kühn) Lopr., *Septoria glumarum* Pass., *Septoria nodorum* (Berk.) Berk. apud Berk. & Br., and *Stagonospora hennebergii* (Kühn) Petr. & Syd (Sutton and Waterston, 1966). This pathogen was recently reviewed by Solomon *et al*. (2006c).

1.2.2.2 Taxonomic placement

The taxonomic placement of *S. nodorum* is as follows: kingdom Fungi; phylum Ascomycota; subphylum Euascomycota; class Dothideomycetes; order Pleosporales; family Phaeosphaeriaceae; genus *Phaeosphaeria*; species *nodorum*. Its genome was recently sequenced and the current assembly consists of a nuclear genome of 37,164,227 bp and a mitochondrial genome of 49,761 bp, with a minimum of 10,762 nuclear genes (Hane *et al.*, 2007). Electrophoretic karyotyping indicated that the nuclear genome is comprised of 14-19 chromosomes (Cooley and Caten, 1991).

1.2.2.3 Host range

In common with necrotrophic pathogens, *S. nodorum* has a broad host range despite a recent claim that it was pathogenic solely on wheat (Prell and Day, 2001). It is a pathogen of bread wheat (*Triticum aestivum* L. subsp. *aestivum*) and related cereals and wild grasses, with infections reported from over 70 species and subspecies in 20 genera (Table 8.1). The fungus was isolated from a further 11 species/subspecies, including an additional 4 genera, although it was not clear from the reports whether growth was pathogenic or epiphytic (Table 8.1).

1.2.2.4 Economic importance

The importance of *S. nodorum* has varied historically and geographically, and since the 1970s it has been overtaken in Europe as the major fungal pathogen of wheat by *Septoria tritici* (Bearchell *et al.*, 2005). It is currently considered to be one of the major diseases of wheat in North America (Singh *et al.*, 2007) and Australia (Solomon *et al.*, 2006c), and is still regarded as being of worldwide significance (Kluge *et al.*, 2006). Crop yields can be significantly reduced by epidemic outbreaks with reported heavy losses ranging from 15% in South Africa (Le Roux, 1984) to 46% in Poland (Pielka, 1957). Australia-wide estimated yield losses have been 18-31% (Bhathal *et al.*, 2003), with higher localised losses of 50% (Loughman *et al.*, 2001) to 70% in reported in Western Australia (Brown and Rosielle, 1980).

1.2.2.5 Nomenclature of the disease

The disease was initially referred to as "septoria" from the then assigned genus of the causal organism (Grove, 1916; Cromwell, 1920; Sutton, 1920). It was subsequently named glume blotch, since the observation of its ability to cause disease symptoms in the glume was used as a major means of distinguishing it from another common wheat pathogen, *Septoria tritici* (Rosen, 1921; Weber, 1922a). However, due to the fact that: (i) this symptom is not always observed; (ii) the taxonomic placement of the organism has undergone several reviews and subsequent name changes; and (iii) some studies have focussed on different components of the disease; there are over

30 names which have been used to describe the disease, with nearly half of these having been employed in publications in the last five years (Table 8.2).

A potential for confusion has been created by terms which describe a disease "complex" caused by two or more independent pathogens (one of which is *S. nodorum*), and by the usage of terms such "Stagonospora leaf blotch disease" to describe the diseases caused by various *Phaeospharia* ssp. (including *P. nodorum*) in a variety of cereals (Ueng *et al.*, 2003; Wang *et al.*, 2007). Since the late 1990s there has been a growing acceptance of the term stagonospora nodorum blotch (SNB), and it is this term which will be used in this study.

1.3 The Infection Process

1.3.1 Life Cycle of Stagonospora nodorum

The life cycle of *Stagonospora nodorum* is depicted in Figure 1.2. Mycelium arising from a germinating spore which has landed on the plant surface or from a seed-borne colony, can penetrate the plant by three methods. Firstly it can exploit natural openings such as stomata or non-natural openings caused by physical damage to the host. Secondly the mycelium can differentiate penetration structures called hyphopodia which can directly penetrate the cell wall. Thirdly, the hyphal tip is also able to directly penetrate the surface of the leaf by breaching the periclinal or anticlinal epidermal cell wall (Solomon *et al.*, 2006f). An apparent host defence response to direct penetration attempts is the deposition of callose at the penetration site, which has been suggested as the target of a β1,3-glucanase produced by the

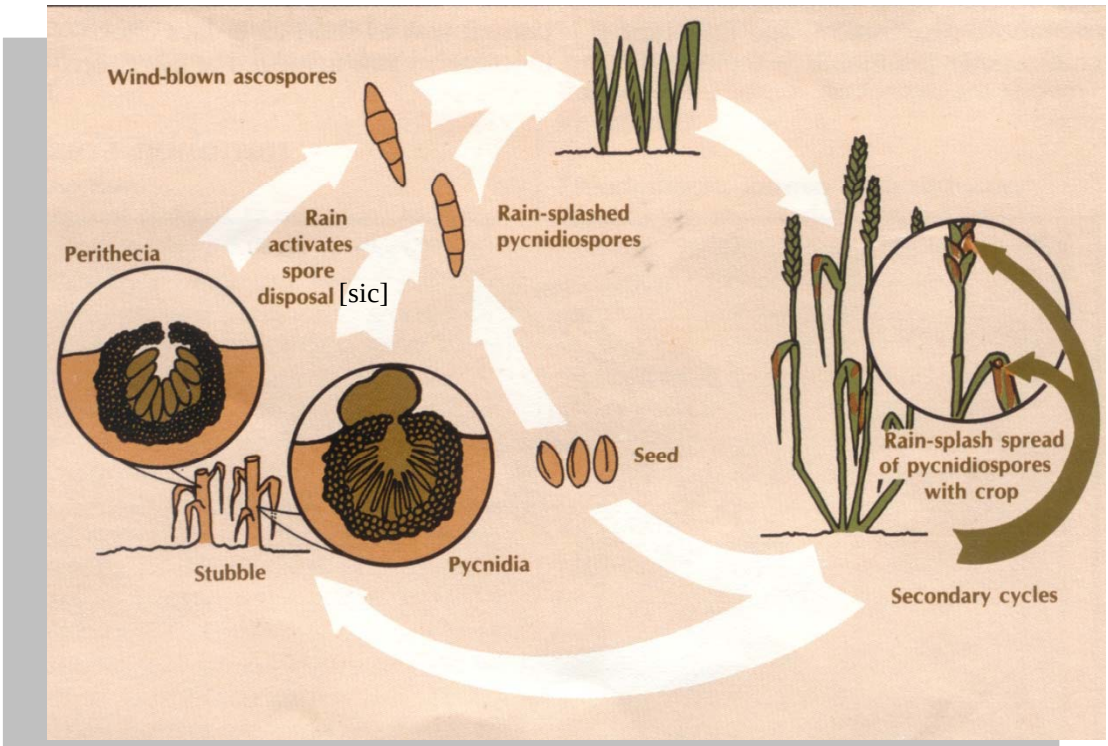

Figure 1.2: The life cycle of *Phaeosphaeria nodorum* [anamorph *Stagonospora nodorum*]. Source: Eyal *et* al. (1987). N.B. “disposal” should read “dispersal”.

pathogen (Lehtinen, 1993). The reported rate of success of direct penetration attempts has varied from 1-5% (Bird and Ride, 1981) to 57% (Solomon *et al.*, 2006f) which most likely reflects differences in the host and pathogen strains used in the different studies and/or the infection conditions.

The period between the initiation of infection and sporulation is referred to as the latent period (Jeger *et al.*, 1984). During this time the mycelium ramifies through the host tissue and initiates a process of host cell destruction resulting in the collapse of the epidermal and mesophyll cells by 6 dpi (Solomon *et al.*, 2006f). Areas of necrotic destruction are macroscopically visible 3-5 days after penetration, appearing as brown oval-shaped lesions. As these increase in size the lesion typically has a light brown to almost translucent, totally necrotic centre with a darker perimeter comprising the active area of ongoing cell destruction. From 5-7 days after initial infection, pycnidia form within the necrotic centre of the lesion. These initially appear beneath the surface of the leaf and subsequently swell to protrude through the leaf surface. The mature pycnidium develops an ostiole which eventually ruptures under the pressure of the pycnidium contents, releasing a pink mucilaginous cirrhus containing pycnidiospores, which are then ready for splash dispersal by rain (Douaiher *et al.*, 2004). The whole process of infection to production of new inoculum takes about 8 days to complete and the potential for serious damage to a wheat crop comes from the polycyclic nature of the disease. With ideal weather conditions, regular bouts of infection can exponentially increase pathogen numbers within a crop.

1.3.2 Modes of Host Inoculation

There are three main sources of primary inoculum for SNB infection. The teleomorph, *Phaeosphaeria nodorum*, produces wind-dispersed ascospores which spread genetic variability within the population and can transport the disease over longer distances (Keller *et al.*, 1997a; Keller *et al.*, 1997b).

Secondly, the anamorph produces asexual pycnidiospores which can survive outside the host growing season in necrotic tissues, volunteers and alternative hosts, and initiate a polycyclic, splash-dispersed infection in the following crop (Eyal *et al.*, 1987).

Thirdly, the fungus can infect the seed, where it can survive as a mycelial colony, causing disease in the sprouted seedling and leading to subsequent pycnidiospore-mediated dispersal to neighbouring plants (Baker, 1970). The fungus has been isolated from harvested seed after 11 years in storage (Cunfer, 1991). Infection rates of 54%-59% of wheat seed have been reported (Cunfer, 1978; Turkington *et al.*, 2002) and without seed treatment this can be a major source of primary inoculum.

1.3.3 Disease Symptoms

The disease is identified in the field by the appearance of chlorotic spots on the leaf although this symptom is similar to that exhibited by infection with *Septoria tritici*. As the disease progresses, the pathogens can be distinguished by the darker

colour of the *S. tritici* pycnidia which give a speckled appearance to the lesion (Weber, 1922a). *Stagonospora nodorum* is also distinguished from *S. tritici* by its ability to infect all above-ground parts of the plant, with *S. tritici* being regarded as limited to infecting leaves (Eyal *et al.*, 1987). However, it has been demonstrated that under controlled conditions, *S. tritici* is also able to cause disease in all above-ground parts of wheat (Jones and Odebunmi, 1971).

1.4 Methods of Disease Control

1.4.1 Chemical Control

The correct application of fungicidal treatments, both in terms of timing and dosage, is a most effective means of controlling outbreaks of disease. Treatment of wheat seed with systemic fungicide can suppress *S. nodorum* for some weeks after germination as the fungicide is translocated from the seed to the leaves (Cunfer, 1993). Treatment with ergosterol inhibitors such triademinol and difenoconazole (Bockus and Shroyer, 1998a) and strobilurin inhibitors of mitochondrial respiration such as azoxystrobin and pyraclostrobin (Jørgensen *et al.*, 1999), or combinations of these, have been shown to significantly suppress disease progress and significantly increase yield (Gaurilčikienė and Ronis, 2006). It has also been reported that these classes of fungicides can induce defence-related genes in wheat (Pasquer *et al.*, 2005). Treatment of seed rather than foliage also has the advantages of more efficient and economic application, and the smaller amount of chemical required reduces any final concentration of resides in the harvest (Bockus and Shroyer, 1998a).

The use of chemical control has a number of limitations:

1. it is a significant expense both to purchase and apply and is not universally economic, especially where the yield is less than 3 tonnes/hectare (Verreet *et al.*, 2000).
2. there are questions regarding the environmental effects of residues
3. it introduces selection pressure, with the capacity for pathogens to adapt to azoxystrobin demonstrated (Morzfeld *et al.*, 2004).

1.4.2 Host Resistance/Tolerance

Host plants usually have some genetic resources which confer resistance or tolerance to a pathogen, and this is considered by some to be the most effective and economic means of achieving durable resistance (Xu *et al.*, 2004b; Singh *et al.*, 2007). Resistance refers to host-pathogen interactions which limit the ability of the pathogen to cause infection, whereas tolerance refers to the ability of the host to maintain its yield and quality in the face of severe infection (Schafer, 1971). Exploiting host resistance is advantageous where it is possible, as there is little cost in terms of application and there is no environmental or dietary implications in terms of chemical residues (Bockus and Shroyer, 1998a). It has been noted, however, that there is a potential yield penalty resulting from use of resistant lines (Oliver *et al.*, 2008b).

Strains of wheat which have exhibited some degree of resistance to *S. nodorum* have been observed or produced by breeding in the past, and recommended for use in areas where the pathogen was prevalent (Brown and McNish, 1974), although the genetic basis of this resistance and the chromosomal location of the

genes involved was not known at the time. It has since been shown that resistance to *S. nodorum* in bread wheat is inherited as a quantitative trait and efforts are focused on the genetic mapping of quantitative trait loci (QTLs) to enable improved breeding for resistance through "Marker Assisted Selection" and gene pyramiding (Servin *et al.*, 2004; Xu *et al.*, 2004b; Uphaus *et al.*, 2007).

1.4.3 Cultural Practices

Prior to the identification of resistant/tolerant strains of wheat and availability of effective and economic fungicides, the disease was controlled through cultural practices designed to reduce the amount of inoculum and minimise the impact of disease on the mature crop. These measures include the avoidance of early maturing varieties and later sowing of crops (Sutton, 1920; Brown and McNish, 1974). Sometimes, however, cultural practises can have antagonistic effects. The growing practise of reduced till/no till agriculture, for the purposes of reducing soil erosion, increasing soil moisture, and conserving energy, has the potential to lead to higher pathogen carryover to the following crop, if crop rotation is not coupled with the practise (Bockus and Shroyer, 1998). A crop rotation of two years between wheat crops in Canada is still considered necessary in reducing disease severity (Duczek *et al.*, 1999), although when environmental conditions are unfavourable to the pathogen, a rotation of one year may be sufficient (Pedersen and Hughes, 1992).

1.4.4 Biological Antagonists

Co-inoculation of *S. nodorum* with field-equivalent densities of the saprophytes *Aureobasidium pullulans*, *Sporobolomyces roseus*, and *Cryptococcus laurentii* var. *flavescens*, is reported to reduce superficial mycelial growth and infection of wheat leaves by *S. nodorum* 50% or more (Fokkema and van der Meulen, 1976).

1.4.5 Genetic Manipulation of S. nodorum

The elucidation of the molecular basis of pathogenicity is considered to be one of the keys to identifying novel antifungal compounds (Divon and Fluhr, 2007). *Stagonospora nodorum* has a haploid genome which has proven amenable to targeted gene disruption, with transformation protocols well established (Solomon *et al.*, 2006c). The release of the *S. nodorum* genome sequence will progress this understanding through comparative genomics. A recent dramatic example of the potential of this approach was the discovery of an operon in *Escherichia coli* consisting of seven previously uncharacterised genes putatively involved in the degradation of nucleic acid precurors (Piškur *et al.*, 2007). In the initial analysis of the *S. nodorum* genome, there were a large number of genes predicted to encode secreted proteins (Hane *et al.*, 2007). The importance of this tool for the isolation and characterisation of these genes cannot be overestimated. To date, a small number of mutants have been produced in *S. nodorum* which have been affected in their ability to initiate and progress SNB in wheat. These include genes involved in metabolism (discussed further below) and signalling pathways.

A gene encoding the Gα sub-unit (*Gna1*) of the heterotrimeric G protein involved in signal transduction was disrupted in *S. nodorum* (Solomon *et al.*, 2004b). While mutants were able to differentiate hyphopodia, they had decreased protease activity and were found to be deficient in their ability to penetrate the cuticle. Although the mutants could still exploit natural openings such as stomata, they were compromised in their ability to cause infection, and were unable to sporulate. A gene (*Mak2*) in the MAP kinase signalling cascade was disrupted in *S. nodorum* (Solomon *et al.*, 2005b) with mutants exhibiting a severely altered phenotype. These were unable to produce hyphopodia and while hyphae were seen to enter via stomata, the infection did not progress and the strains were essentially non-pathogenic on wheat. The strain was also unable to sporulate either *in planta* or *in vitro*. These two mutants in different signalling pathways underscore the importance of signalling to the infection process.

1.5 Metabolism and Infection

1.5.1 Definition of Metabolism

Metabolism refers to the operation and integrative functioning of the complex of metabolic pathways which are required by an organism in order to complete its life cycle (Vining, 1990). As such it encompasses the anabolic and catabolic manipulation of molecules, together with the regulation of those processes. There is a distinction between primary and secondary metabolism. Primary metabolism defines those pathways which are essential to the basic growth, development and reproduction of an organism (Mann, 1978). These pathways and metabolites are common to all organisms (Vining, 1992), although some major differences occur between prokaryotes and eukaryotes. Secondary metabolism defines the pathways involved in the production of a vast array of metabolites that are operationally characterised by being non-essential for growth, specific to particular organisms, and have a wide range of structures and activities (Idnurm and Howlett, 2001). This differentiation is artificial and does not imply that pathways of one class are unrelated to, or do not interact with, pathways of the other class. There are also some which can appear in both classes.

1.5.2 The Impact of Disrupted Metabolism on Infection

There are a number of *S. nodorum* mutants which have been produced in which genes involved in metabolism have been disrupted. These include some which

have been affected in their virulence or pathogenicity during the three main stages of infection.

1.5.2.1 Germination and penetration

The early stages of infection require the pathogen spore to germinate, recognise the host and initiate metabolism of cell wall degrading enzymes and proteases. The glyoxylate cycle gene malate synthase (*Mls1*) was deleted and mutant spores found to be incapable of germination without the addition of exogenous glucose or sucrose (Solomon *et al.*, 2004a). This implied that germination and infection are dependent upon lipid catabolism in order for the glyoxylate cycle and gluconeogenesis to mobilise energy stores for germination.

The secretion of cell wall degrading enzymes is characteristic of necrotrophic fungi as a means of penetrating host cells and reducing their contents for nutrient uptake (Oliver and Ipcho, 2004). The cuticle of plants consists of a complex of compounds designed to protect it from abiotic stress and biotic attack. *Stagonospora nodorum* produces a wide variety of these including xylanases, polygalacturonases, glucanases, xylosidases, glucosidases and galactosidase, most of them with multiple isoenzymes (Lehtinen, 1993), as well as a trypsin-like protease (SNP1) (Carlile *et al.*, 2000). Enzyme family genes are poor candidates for disruption, since the disruption of one member of the family leaves the others unaffected. Disruption of the *Snp1* gene abolished trypsin activity, but the mutants were unaffected in pathogenicity since an unknown alkaline protease compensated for the disrupted gene (Bindschedler *et al.*, 2003).

1.5.2.2 Proliferation

Following the initial stage of infection, the pathogen must obtain nutrients from the host in order to enable it to proliferate, to continue to produce degradative molecules, and counteract any further host defence responses. A 3-isopropylmalate dehydrogenase mutant of *S. nodorum* which was auxotrophic for leucine and lost pathogenicity to wheat, added to evidence that basic biosynthetic pathways can contribute to pathogenicity (Cooley *et al.*, 1999). The di/tripeptide transporter gene (*Ptr2*), which is upregulated during early infection and is solely responsible for uptake of degraded peptides, was abolished but without any effect on pathogenicity (Solomon *et al.*, 2003). Targeted deletion of the gene encoding 5-aminolevulinate synthase (*Als1*) produced a mutant which was auxotrophic for δ-aminolaevulinic acid, a precursor in the synthesis of haem (Solomon *et al.*, 2006a). Mutant strains had severely stunted germ tubes and became unviable even upon wounded leaves, indicating a dependence on exogenous stores and synthesis of the compound. Disruption of the ornithine decarboxylase (*Odc1*) gene which converts ornithine to the polyamine putrescine, required for cell division and resistance to oxidative stress, resulted in mutants with reduced virulence (Bailey *et al.*, 2000). The emerging picture from these studies is that whilst the fungus apparently has access to large reserves of host resources at this time, it is still dependent upon its own ability to synthesise many essential primary metabolites from simple precursors.

Secondary metabolites such as host-specific toxins are important determinants of host range in pathogens. The *ToxA* gene of *S. nodorum* confers pathogen virulence to wheat varieties containing the *Tsn1* gene and culture filtrates from strains in which

ToxA had been ablated were non-toxic (Friesen *et al.*, 2006). Further studies have shown the presence of multiple *S. nodorum* toxins each of which are required to interact with specific wheat gene products in an inverse gene-for-gene fashion in order to cause disease (Friesen *et al.*, 2007). This is an exciting new area of research with immediate applicability in the field.

1.5.2.3 Sporulation

Sporulation completes the life cycle of the phytopathogenic fungus and as noted above, signalling pathways play an important role in this process. An investigation of the calcium/calmodulin-dependent protein kinases in *S. nodorum* demonstrated that while *CpkB* was redundant for pathogenicity, disruption of the *CpkA* gene resulted in an inability to complete differentiation of pycnidia, while disruption of the *CpkC* gene resulted in delayed lesion development and sporulation (Solomon *et al.*, 2006d).

The disaccharide trehalose was found to be upregulated during sporulation and the gene encoding trehalose 6-phosphate synthase (*Tps1*) was inactivated by targeted gene deletion (Lowe, 2006). Mutants were almost completely deficient in trehalose accumulation, and while lesion development was only slightly affected, sporulation was reduced to 30% of wild type levels.

Stagonospora nodorum mutants in which the mannitol 1-phosphate 5-dehydrogenase (*Mpd1*) gene was disrupted were phenotypically similar to the wild type except that they were unable to sporulate *in planta* (Solomon *et al.*, 2005a). A

second gene involved in mannitol metabolism, *Mdh1*, which encodes mannitol 2-dehydrogenase, was subsequently inactivated in *S. nodorum* (Waters, 2004), and found to be phenotypically identical to the wild type. The observed behaviours of these two mutants implied a role in pathogenicity for the compound itself, and also contradicted the accepted theory of how mannitol metabolism occurs as outlined below.

1.6 Mannitol Metabolism and Infection

1.6.1 Postulated Roles of Mannitol

The acyclic 6-carbon polyol D-mannitol is the most abundant soluble metabolite found in many filamentous fungi (Lewis and Smith, 1967). There have been a number of postulated roles for mannitol, not necessarily mutually exclusive, including:

- Carbohydrate storage (Birkinshaw *et al.*, 1931; Corina and Munday, 1971)
- Translocation of carbon (Trip *et al.*, 1964; Lewis and Smith, 1967; Koide *et al.*, 2000)
- Compatible solute/protein protection (Stoop and Mooibroek, 1998; Ortbauer and Popp, 2008)
- Storage of reducing power (Lewis and Smith, 1967; Ruijter *et al.*, 2003)
- Co-enzyme regulation/NADPH regeneration (Hult and Gatenbeck, 1978; Diano *et al.*, 2006)
- Energy dissipation by futile cycling (Jennings and Burke, 1990)

- Morphogenesis/conidiation/spore discharge (Corina and Munday, 1971; Webster *et al.*, 1995; Trail *et al.*, 2005)
- Environmental stress, including quenching of reactive oxygen species produced as a defence response by the host (Chaturvedi *et al.*, 1996; Stoop and Mooibroek, 1998; Ruijter *et al.*, 2003; Bois *et al.*, 2006)
- Host carbon sequestration (Joosten *et al.*, 1990; Noeldner *et al.*, 1994)

These last three imply a specific role for mannitol in the process of infection. While there is circumstantial evidence which supports some of the above roles, almost none have been experimentally proven. Mannitol is not the sole candidate compound for some of these roles - other polyols have better experimental support for some roles in different fungal species (see Solomon *et al*. (2007) for a summary of these arguments) - and the absence of mannitol in some species (Lewis and Smith, 1967), particularly laboratory strains of *Saccharomyces cerevisiae*, indicates that alternative compounds must be able to compensate.

1.6.2 Enzymatic Metabolism of Mannitol in Fungi

There are a number of enzymes which have been demonstrated or purported to be involved in the metabolism of mannitol in fungi. A summary of the organisms in which these enzymes have been reported is given in Table 1.3 and the enzymes are outlined below.

Table 1.3: Distribution of genes reported as being involved in the metabolism of mannitol in fungal species (see below for abbreviations and notes).

Species	Phylum[2]	Hex	F6PP	NADP-Mdh	NAD-Mdh	Mdh	M1Pdh	M1PP	MK	MPPT	MAPT	References
Alternaria alternata	A	Y	-	Y	N	-	Y	Y	-	-	-	(Hult and Gatenbeck, 1978; Hult and Gatenbeck, 1979; Hult *et al.*, 1980; Schneider *et al.*, 2006; Vélëz *et al.*, 2007)
Aspergillus candidus	A	Y	-	Y	N	-	Y	Y	N	-	-	(Strandberg, 1969)
Aspergillus clavatus	A	-	-	-	-	-	Y	-	-	-	-	(Corina and Munday, 1971)
Aspergillus fumigatus	A	-	-	-	-	-	Y	-	-	-	-	(Boonsaeng *et al.*, 1976)
Aspergillus nidulans	A	Y	-	Y	-	-	Y	Y	-	-	-	(Hankinson, 1974; Bailey and Arst, 1975; Hankinson and Cove, 1975; Singh *et al.*, 1988)
Aspergillus niger	A	Y	-	Y	Y	-	Y	Y	-	-	-	(Yamada *et al.*, 1959; Boonsaeng *et al.*, 1976; Hult *et al.*, 1980; Kiser and Niehaus, 1981; Foreman and Niehaus, 1985; Ruijter *et al.*, 2003)
Aspergillus oryzae	A	-	-	Y	Y	-	Y	Y	-	-	-	(Yamada *et al.*, 1959; Horikoshi *et al.*, 1965; Boonsaeng *et al.*, 1976; Ruijter *et al.*, 2004)
Aspergillus parasiticus	A	-	-	Y	-	-	Y	-	-	-	-	(Niehaus and Dilts, 1982; Buchanan and Lewis, 1984b; Buchanan and Lewis, 1984a; Foreman and Niehaus, 1985; Niehaus and Jiang, 1989)
Aspergillus sp. (UC4177)[1]	A	Y	-	N	N	-	Y	Y	N	N	Y	(Lee, 1967a; Lee, 1967b; Lee, 1970)
Botrytis cinerea	A	Y	-	Y	N	-	Y	Y	-	-	-	(Hult *et al.*, 1980)
Candida magnoliae	A	-	-	Y	-	-	-	-	-	-	-	(Lee *et al.*, 2003)
Candida utilis	A	Y	-	Y	N	-	N	Y	-	-	-	(Hult *et al.*, 1980)

Continued on following page

Table 1.3: Distribution of genes reported as being involved in the metabolism of mannitol in fungal species (cont.) - (see below for abbreviations and notes).

Species	Phylum[2]	Hex	F6PP	NADP-Mdh	NAD-Mdh	Mdh	M1Pdh	M1PP	MK	MPPT	MAPT	References
Cenococcum graniforme	A	Y[3]	-	Y	Y	-	Y[9]	-	-	-	-	(Martin *et al.*, 1985)
Ceratocystis multiannulata	A	Y	-	Y	N	-	N	Y	-	-	-	(Hult *et al.*, 1980)
Chaetomium globosum	A	Y[3]	-	N	Y	-	N[9]	-	N	N	N	(Adomako *et al.*, 1972)
Chaetomium thermophile var. *dissitum*[1]	A	-	-	-	-	-	Y	-	-	-	-	(Boonsaeng *et al.*, 1976)
Cladosporium cladosporioides	A	Y	-	Y	Y	-	Y	Y	-	-	-	(Hult *et al.*, 1980)
Cladosporium fulvum	A	-	-	Y	-	-	-	-	-	-	-	(Noeldner *et al.*, 1994)
Cladosporium herbarum	A	-	-	Y	-	-	-	-	-	-	-	(Simon-Nobbe *et al.*, 2006)
Coccidioides immitis	A	-	-	-	-	N	Y	N	N	-	-	(Lones and Peacock, 1964)
Dendryphiella salina	A	-	-	Y	Y	-	N[9]	-	-	-	-	(Holligan and Jennings, 1972)
Diplodia viticola	A	-	-	Y	N	-	-	-	-	-	-	(Strobel and Kosuge, 1965)
Geotrichum candidum	A	-	-	Y	-	-	-	-	-	-	-	(Chang and Li, 1964)
Gibberella zeae	A	Y	-	Y	N	-	N	Y	-	-	-	(Hult *et al.*, 1980; Trail and Xu, 2002; Trail *et al.*, 2002)
Hypogymnia physodes	A	-	-	-	-	-	Y	-	-	-	-	(Jensen *et al.*, 1991)
Magnaporthe syn. *Pyricularia oryzae*	A	Y	-	Y/N	Y/N	N	Y	Y	-	-	-	(Yamada *et al.*, 1959; Yamada *et al.*, 1961; Hult *et al.*, 1980)
Malbranchea pulchella var. *sulfurea*	A	-	-	-	-	-	Y	-	-	-	-	(Boonsaeng *et al.*, 1976)
Microsporum gypseum	A	Y[3,4]	-	Y	-	-	Y	Y	Y	Y	-	(Leighton *et al.*, 1970)
Neurospora crassa	A	Y	-	Y	Y	-	N[10]	Y	-	-	-	(Yamada *et al.*, 1959; Hult *et al.*, 1980)
Neurospora sitophila	A	-	-	-	-	-	N	Y	-	-	-	(Yamada *et al.*, 1959)

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Table 1.3: Distribution of genes reported as being involved in the metabolism of mannitol in fungal species (cont.) - (see below for abbreviations and notes).

Species	Phylum[2]	Hex	F6PP	NADP-Mdh	NAD-Mdh	Mdh	M1Pdh	M1PP	MK	MPPT	MAPT	References
Paracoccidioides brasiliensis	A	-	-	-	-	-	Y	-	-	-	-	(Castro *et al.*, 2002)
Penicillium chrysogenum syn. *notatum*	A	-	-	-	Y	-	Y	Y	-	-	-	(Ballio *et al.*, 1964; Boonsaeng *et al.*, 1976; Boonsaeng *et al.*, 1977; Boutelje *et al.*, 1983)
Penicillium cyclopium	A	-	-	-	-	-	Y	-	-	-	-	(Boonsaeng *et al.*, 1976)
Penicillium duponti[1]	A	-	-	-	-	-	Y	-	-	-	-	(Boonsaeng *et al.*, 1976; Boonsaeng *et al.*, 1977)
Penicillium glabrum syn. *frequentans*	A	Y	-	Y	N	-	Y	Y	-	-	-	(Hult *et al.*, 1980)
Penicillium islandicum	A	Y	-	Y	Y	-	Y	Y	-	-	-	(Hult *et al.*, 1980)
Penicillium urticae	A	-	-	-	-	-	Y	-	-	-	-	(Boonsaeng *et al.*, 1976)
Pseudevernia furfuracea	A	-	-	-	-	-	Y	-	-	-	-	(Jensen *et al.*, 1991)
Pyrenochaeta terrestris	A	-	-	-	-	-	Y	Y	-	-	-	(Wright and Le Tourneau, 1966; Aitken *et al.*, 1969a; Aitken *et al.*, 1969b)
Saccharomyces cerevisiae	A	-	-	Y	Y	-	-	-	-	-	-	(Kulbe *et al.*, 1986; Quain and Boulton, 1987; Perfect *et al.*, 1996)
Sclerotinia sclerotiorum	A	-	-	Y	-	-	Y	Y	-	-	-	(Wang and Le Tourneau, 1972)
Sphaerosporella brunnea	A	Y	-	Y	Y	-	Y	Y	-	-	-	(Ramstedt *et al.*, 1987)
Stagonospora nodorum	A	-	-	Y	-	-	Y	-	N[11]	-	-	(Solomon *et al.*, 2005; Solomon *et al.*, 2006b)
Trichothecium roseum	A	Y	-	Y	Y	-	Y	Y	-	-	-	(Hult *et al.*, 1980)

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Table 1.3: Distribution of genes reported as being involved in the metabolism of mannitol in fungal species (cont.) - (see below for abbreviations and notes).

Species	Phylum[2]	Hex	F6PP	NADP-Mdh	NAD-Mdh	Mdh	M1Pdh	M1PP	MK	MPPT	MAPT	References
Thermomyces lanuginosus syn. *Humicola lanuginosa*	A	Y	-	Y	Y	-	Y	Y	-	-	-	(Boonsaeng *et al.*, 1976; Hult *et al.*, 1980)
Tuber borchii	A	Y[5]	-	Y	-	-	Y	Y	-	-	-	(Ceccaroli *et al.*, 1999; Ceccaroli *et al.*, 2003; Ceccaroli *et al.*, 2007)
Agaricus bisporus	B	Y[6]	Y	Y	N	-	N	N	-	-	-	(Hammond, 1977; Ruffner *et al.*, 1978; Hult *et al.*, 1980; Hammond, 1981; Morton *et al.*, 1985a; Morton *et al.*, 1985b; Stoop and Mooibroek, 1998; Wannet *et al.*, 1999; Hörer *et al.*, 2001; Sassoon *et al.*, 2001; Sassoon and Mooibroek, 2001)
Agaricus campestris	B	-	-	Y	Y	-	-	-	-	-	-	(Edmundowicz and Wriston, 1963)
Amanita muscaria	B	Y	-	Y	Y	-	N	Y	-	-	-	(Ramstedt *et al.*, 1987)
Armillariella mellea	B	Y	-	N	N	-	N	N	-	-	-	(Ramstedt *et al.*, 1987)
Chondrostereum purpureum	B	Y	-	N	Y	-	N	N	-	-	-	(Ramstedt *et al.*, 1987)
Coprinus cinereus	B	-	-	-	Y	-	-	-	-	-	-	(Nyunoya *et al.*, 1984)
Cryptococcus neoformans	B	-	-	Y	Y	-	Y	-	-	-	-	(Perfect *et al.*, 1996; Suvarna *et al.*, 2000; Loftus *et al.*, 2005)
Flammulina velutipes	B	-	-	N	Y	-	N	-	-	-	-	(Kitamoto *et al.*, 2000)
Fomes pinicola	B	Y	-	N	Y	-	N	N	-	-	-	(Hult *et al.*, 1980)
Heterobasidion annosum	B	Y	-	N	Y	-	N	N	-	-	-	(Ramstedt *et al.*, 1987)

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Table 1.3: Distribution of genes reported as being involved in the metabolism of mannitol in fungal species (cont.) - (see below for abbreviations and notes).

Species	Phylum[2]	Hex	F6PP	NADP-Mdh	NAD-Mdh	Mdh	M1Pdh	M1PP	MK	MPPT	MAPT	References
Laccaria laccata	B	Y	-	Y	N	-	N	Y	-	-	-	(Ramstedt *et al.*, 1987; Deveau *et al.*, 2008)
Lentinus edodes	B	Y[7]	Y	Y	-	-	N	Y	-	-	-	(Kulkarni, 1990)
Marasmius scorodonius	B	Y	-	N	Y	-	N	Y	-	-	-	(Ramstedt *et al.*, 1987)
Melampsora lini	B	-	-	Y	Y	-	-	-	-	-	-	(Clancy and Coffey, 1980)
Mycena metata	B	Y	-	N	Y	-	N	Y	-	-	-	(Ramstedt *et al.*, 1987)
Phanerochaete chrysosporium	B	-	-	-	-	-	Y	-	-	-	-	(Matsuzaki *et al.*, 2008)
Piloderma croceum	B	Y	-	Y	Y	-	N	Y	-	-	-	(Ramstedt *et al.*, 1986; Ramstedt *et al.*, 1987)
Pisolithus tinctorius	B	-	-	Y	-	-	-	-	-	-	-	(Kong *et al.*, 2000)
Pleurotus ostreatus	B	Y[7]	Y	-	-	Y	Y	Y	-	-	-	(Chakraborty *et al.*, 2003; Chakraborty *et al.*, 2004)
Polyporus versicolor	B	Y	-	N	N	-	N	N	-	-	-	(Hult *et al.*, 1980)
Puccinia graminis	B	-	-	-	Y	-	-	-	-	-	-	(Maclean, 1971)
Schizophyllum commune	B	-	-	-	Y	-	-	-	-	-	-	(Niederpruem *et al.*, 1965; Isenberg and Niederpruem, 1967)
Suillus bovinus	B	Y	-	Y	N	-	N	N	-	-	-	(Ramstedt *et al.*, 1987)
Suillus variegatus	B	Y	-	Y	N	-	N	Y	-	-	-	(Ramstedt *et al.*, 1987)
Uromyces phaseoli[1]	B	-	-	Y	Y	-	-	-	-	-	-	(Wynn, 1966)
Uromyces viciae-fabae syn. *fabae*	B	-	-	Y	-	-	-	-	-	-	-	(Voegele *et al.*, 2005)
Absidia glauca	IS	-	-	-	Y	-	Y	-	Y	-	-	(Ueng *et al.*, 1976; Ueng and McGuinness, 1977)
Amylomyces syn. *Mucor rouxii*	IS	-	-	-	-	-	Y	-	-	-	-	(Boonsaeng *et al.*, 1976)

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Table 1.3: Distribution of genes reported as being involved in the metabolism of mannitol in fungal species (cont.) - (see below for abbreviations and notes).

Species	Phylum[2]	Hex	F6PP	NADP-Mdh	NAD-Mdh	Mdh	M1Pdh	M1PP	MK	MPPT	MAPT	References
Cephalosporium chrysogenum?	IS	-	-	Y	-	-	-	-	-	-	-	(Birken and Pisano, 1976)
Mucor circinelloides f. lusitanicus	IS	Y	-	Y	Y	-	N	N	-	-	-	(Hult *et al.*, 1980)
Mucor genevensis[1]	IS	-	-	-	-	-	N	-	-	-	-	(Boonsaeng *et al.*, 1976)
Phycomyces blakesleeanus	IS	Y	-	N	N	-	N	N	-	-	-	(Hult *et al.*, 1980)
Rhizomucor syn. *Mucor miehei*	IS	-	-	-	-	-	N	-	-	-	-	(Boonsaeng *et al.*, 1976)
Rhizomucor syn. *Mucor pusillus*	IS	-	-	-	-	-	N	-	-	-	-	(Boonsaeng *et al.*, 1976)
Rhizopus arrhizus	IS	Y	-	N	N	-	N	N	-	-	-	(Hult *et al.*, 1980)

Abbreviations: Hex – hexokinase; F6PP – fructose 6-phosphate phosphatase; NADP-Mdh – $NADP^+$-dependent mannitol dehydrogenase (EC 1.1.1.138); NAD-Mdh – NAD^+-dependent mannitol dehydrogenase (EC 1.1.1.67); Mdh – mannitol dehydrogenase (co-factor not specified); M1Pdh – mannitol 1-phosphate dehydrogenase (EC 1.1.1.17); M1PP – mannitol-1-phosphate phosphatase (EC 3.1.3.22); MK – mannitol kinase; MPPT – mannitol phosphoenolpyuvate phosphotransferase; MAPT – mannitol acyl phosphotransferase; A – ascomycota; B – basidiomycota; IS – fungi incertae sedis; Y = activity detected; N = activity not detected; Y/N = activity present in some studies but not in others; - = activity not assayed.

[1] Organisms not found in Taxbrowser database (NCBI, 2008). Names have been given as reported.

[2] Some organisms were originally reported from obsolete phyla e.g. phycomyces. The currently accepted phylum as assigned in Taxbrowser (NCBI, 2008) is used here. In the case of organisms noted in the previous note – these were assigned to phyla based on their genus.

[3] Reported a separate fructokinase activity in addition to hexokinase

[4] Glucokinase activity was reported to be greater than fructokinase activity

[5] Three types of hexokinase activity reported, expressed at different stages of development

[6] Three studies report hexokinase activity, one study reports fructokinase activity

[7] Only reported fructokinase activity

[8] One study reports hexokinase, the other specifies fructokinase

[9] Both co-factors assayed

[10] A gene identified as M1PDH was reported in the genome sequence of *Neurospora crassa* (Galagan *et al.*, 2003)

[11] pers. comm., Dr. P. Solomon

1.6.2.1 D-mannitol:NADP$^+$ 2-oxidoreductase (EC 1.1.1.138)

Synonyms: mannitol 2-dehydrogenase (NADP); mannitol 2-dehydrogenase (NADP$^+$); NADP-mannitol dehydrogenase; mannitol dehydrogenase (the last of these will be used hereafter).

Reaction:

$$\text{D-fructose} + \text{NADPH} + \text{H}^+ \Longleftrightarrow \text{D-mannitol} + \text{NAPD}^+$$

The first identification of this enzyme in fungi resulted from an investigation into mannitol metabolism in the basidiomycete *Agaricus campestris* (Edmundowicz and Wriston, 1963). It was first purified and characterised in *Aspergillus parasiticus* (Niehaus and Dilts, 1982). In *Aspergillus nidulans*, this enzyme was shown to be localised exclusively in the cytosol (Singh *et al.*, 1988). The enzyme was first inactivated by gene disruption in *Stagonospora nodorum* (Waters, 2004) and mutants found to be phenotypically identical to the wild type.

1.6.2.2 D-mannitol:NAD$^+$ 2-oxidoreductase (EC 1.1.1.67)

Synonyms: mannitol 2-dehydrogenase; D-mannitol dehydrogenase; mannitol dehydrogenase; NAD-mannitol dehydrogenase (the last of these will be used hereafter)

Reaction:

$$\text{D-fructose} + \text{NADH} + \text{H}^+ \Longleftrightarrow \text{D-mannitol} + \text{NAD}^+$$

In cases where NAD-mannitol dehydrogenase has been detected in addition to the NADP-linked mannitol dehydrogenase, its activity has been lower (5-10% in *A. campestris* and 25% in *Aspergillus oryzae*) than that of the NADP-linked enzyme (Edmundowicz and Wriston, 1963; Horikoshi *et al.*, 1965).

1.6.2.3 D-mannitol-1-phosphate:NAD$^+$ 5-oxidoreductase (EC 1.1.1.17)

Synonyms: hexose reductase; mannitol-1-phosphate 5-dehydrogenase; D-mannitol-1-phosphate dehydrogenase; fructose 6-phosphate reductase; mannitol 1-phosphate dehydrogenase (the last of these will be used hereafter)

Reaction:

D-fructose 6-phosphate + NADH + H^+ $\Longleftrightarrow$ D-mannitol 1-phosphate + NAD^+

Activity corresponding to this enzyme was first reported in fungi in *Magnaporthe* (syn. *Pyricularia*) *oryzae*, *A. oryzae* and *A. niger* (Yamada *et al.*, 1959). It was purified and characterised in terms of its kinetic parameters in *A. niger* and found to be highly specific for its substrates and co-factors (Kiser and Niehaus, 1981). Two isoenzymes were detected in *A. nidulans*, one of which was localised in the cytosol, and the other apparently present on the outer face of the inner mitochondrial membrane (Singh *et al.*, 1988). The *MpdA* gene encoding this enzyme in the saprobe *A. niger* was inactivated, with a reduction of mannitol concentration in conidiospores to 30% of wild type levels (Ruijter *et al.*, 2003). Conidiospores were found to be extremely sensitive to heat stress and oxidative stress, demonstrating a role for

mannitol in this non-pathogen species. The *mpd1* mutants of *S. nodorum* had mannitol reduced to 20% of the wild type, and lost the ability to sporulate *in planta*, the first demonstrated role for mannitol metabolism in pathogenicity (Solomon *et al.*, 2005a).

1.6.2.4 D-mannitol-1-phosphate phosphohydrolase (EC 3.1.3.22)

Synonyms: mannitol-1-phosphatase; mannitol-1-phosphate phosphatase (the last of these will be used hereafter).

Reaction:

$$\text{D-mannitol 1-phosphate} + H_20 \quad \Longleftrightarrow \quad \text{D-mannitol} + P_i$$

Activity corresponding to this enzyme was first reported in fungi in *M. oryzae* and activity also noted in the crude extracts of *A. oryzae, A. niger*, *Neurospora crassa* and *N. sitophila* (Yamada *et al.*, 1959). It has been shown to be localised exclusively in the cytosol in *A. nidulans* (Singh *et al.*, 1988).

1.6.2.5 D-mannitol kinase (EC 2.7.1.57 (created 1972, deleted 1984))

Reaction (theoretical):

$$\text{D-mannitol} + \text{ATP} \rightarrow \text{D-mannitol 1-phosphate} + \text{ADP}$$

While evidence of activity corresponding to this enzyme was reported for *Absidia glauca* (Ueng *et al.*, 1976) and *Microspora gypseum* (Leighton *et al.*, 1970), in the majority of studies where such an enzyme was investigated in fungi, there was

no activity reported (Lones and Peacock, 1964; Lee, 1967b; Strandberg, 1969; Adomako *et al.*, 1972). Mannitol kinase activity was reported in a number of bacteria (Klungsøyr, 1966; Mehta *et al.*, 1977), however, no gene has been forwarded as a candidate for transcription of this enzyme, and experimental evidence for its existence is poor. It is possible that there are one or more non-specific kinases which could contribute independently or in concert to such an activity.

1.6.2.6 D-mannitol acetyl phosphate phosphotransferase (no EC number)

Activity corresponding to this enzyme was reported in an *Aspergillus* strain by which mannitol was phosphorylated to mannitol 1-phosphate (Lee, 1967b). Acetyl phosphate and carbamyl phosphate were both able to serve as phosphate donors in the reaction. No activity for this enzyme was found in *Chaetomium globosum* (Adomako *et al.*, 1972).

1.6.2.7 D-mannitol phosphoenolpyruvate phosphotransferase (no EC number)

This enzyme is considered to be part of the bacterial system of mannitol catabolism (Ramstedt *et al.*, 1986). There is a single report of activity corresponding to this enzyme system in fungi in *M. gypseum* (Leighton *et al.*, 1970). No activity for this enzyme was found in *C. globosum* (Adomako *et al.*, 1972) or *Aspergillus* (Lee, 1967b).

1.6.2.8 Hexokinases

This group of enzymes participates in the pathway of mannitol metabolism by phosphorylating fructose to fructose 6-phosphate, or glucose to glucose 6-phosphate (the glycolytic precursor of fructose 6-phosphate) and thereby providing the substrate for mannitol 1-phosphate dehydrogenase. The term may refer to any or all of three enzymes recognised by the Enzyme Commission. These are glucokinase (EC 2.1.1.2) which is specific for conversion of glucose to glucose 6-phosphate; fructokinase (EC 2.1.1.4) which is specific for conversion of fructose to fructose 6-phosphate; and hexokinase (EC 2.1.1.1) which can catalyse both reactions.

1.6.2.9 D-fructose-6-phosphate phosphatase (no EC number)

Synonyms: fructose-6-phosphatase

Reaction:

D-fructose 6-phosphate + H_2O → D- fructose + Pi

Perhaps due to a general acceptance of the mannitol cycle (described below) and its unidirectional operation, there has been less attention given to this reaction which reverses the hexokinase-catalysed step. Activity corresponding to this enzyme has been reported in three basidiomycetes – *Agaricus bisporus* (Morton *et al.*, 1985b), *Lentinus edodes* (Kulkarni, 1990) and *Pleurotus ostreatus* (Chakraborty *et al.*, 2004). The enzyme has not yet been purified and characterised, and there is no gene put forward as encoding such an enzyme.

1.6.3 The Postulated Mannitol Cycle

The proposition that the metabolism of mannitol in fungi occurred in a unidirectional enzymatic cycle was first proposed by Hult & Gatenbeck (1978). In this cycle (Figure 1.3A) fructose 6-phosphate was converted to mannitol 1-phosphate by mannitol 1-phosphate dehydrogenase, with the latter compound dephosphorylated to mannitol by mannitol 1-phosphate phosphatase. Mannitol was then converted to fructose by mannitol dehydrogenase, and in the final step fructose was phosphorylated to fructose 6-phosphate by hexokinase (Hult and Gatenbeck, 1978). The net result of the cycle was the stoichiometric regeneration of NADPH at the expense of NADH and ATP, and NADPH regeneration was given as the main purpose of the cycle. Since the mannitol 1-phosphate phosphatase-catalysed reaction was assumed to be irreversible, the cycle could only proceed in one direction. A variation of this cycle was proposed in which the direct conversion of fructose to fructose 6-phosphate was replaced by several steps involving the conversion of fructose to glucitol (sorbitol), followed by glucose, glucose 6-phosphate, and then fructose 6-phosphate (Jennings and Burke, 1990) (Figure 1.3b).

The existence and importance of the mannitol cycle gained steady acceptance (Martin *et al.*, 1985; Niehaus and Jiang, 1989; Schmatz *et al.*, 1989; Michalski *et al.*, 1992; Schmidt *et al.*, 1998; Allocco *et al.*, 1999; Ceccaroli *et al.*, 2003) in the absence of a means of scientifically falsifying the theory. There was some questioning of the fact and significance of the cycle based on subcellular location of the enzymes, and an observed lack of coordination in their maximal activities in *Aspergillus nidulans*

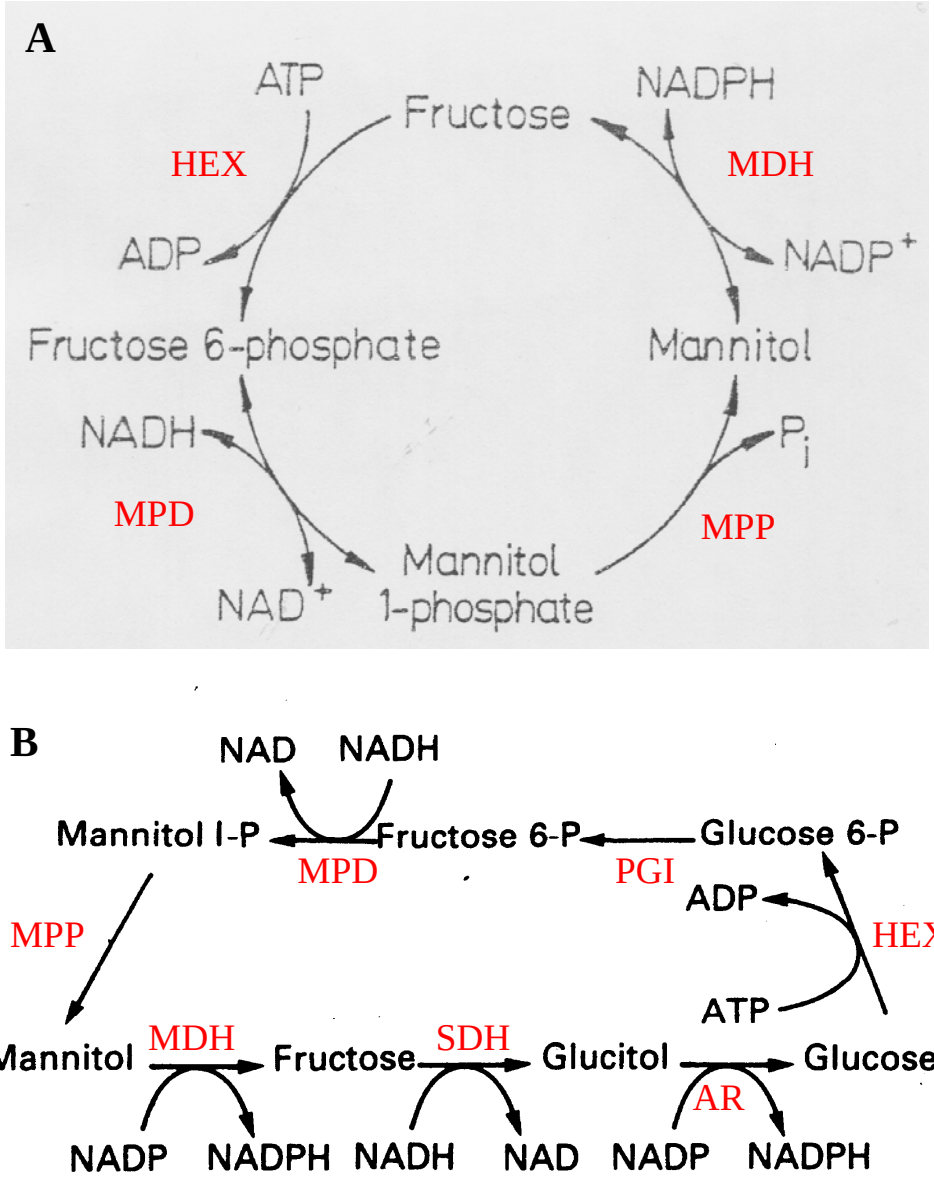

Figure 1.3: (A) The mannitol enzymatic cycle as proposed by Hult and Gatenbeck (1978). Figure as given in Hult *et al*. (1980).

(B) The modified mannitol cycle proposed by Jennings and Burke (1990).

The enzymes for each step have been added in red. Abbreviations: AR = aldose reductase (EC 1.1.1.21); HEX = hexokinase; MDH = mannitol dehydrogenase (EC 1.1.1.138); MPD = mannitol 1-phosphate dehydrogenase (EC 1.1.1.67); MPP = mannitol-1-phosphate phosphatase; PGI = phosphoglucose isomerase; SDH = sorbitol (glucitol) dehydrogenase (EC 1.1.1.14). Note the proposed unidirectional operation of the cycles.

(Singh *et al.*, 1988). The involvement of NADH in a synthetic reaction was questioned, as was the need for a pathway of NADPH re-generation in addition to that of the pentose phosphate pathway, and the fact that the cycle required fructose to be a better substrate for hexokinase than glucose (Jennings and Burke, 1990). Furthermore, the proposers of the cycle themselves were among those who examined a range of fungal species for existence of the cycle and found that basidiomycetes in general did not have the full complement of enzymes (Hult *et al.*, 1980; Ramstedt *et al.*, 1987; Kulkarni, 1990; Kitamoto *et al.*, 2000; Deveau *et al.*, 2008). Mannitol 1-phosphate dehydrogenase activity was not detected in basidiomycetes in any of these studies and mannitol 1-phosphate phosphatase was not detected in the majority. The only reported exceptions to this were:

1. *Pleurotus ostreatus*, in which all enzymes of the proposed cycle were detected (Chakraborty *et al.*, 2004)
2. *Cryptococcus neoformans*, from which Mpd1 was purified by Suvarna *et al*. (2000), but the presence of other enzymes of the mannitol cycle was not investigated. The recently released genome sequence contains two genes reported as encoding Mpd1 (Loftus *et al.*, 2005)
3. *Phanerochaete chrysosporium*, in which Mpd1 was found to be upregulated following growth on benzoic acid (Matsuzaki *et al.*, 2008)

The reported presence in many fungal species, of an NAD^+-dependent mannitol dehydrogenase, instead of, or in addition to, the $NADP^+$-dependent enzyme required for the operation of the cycle (Table 1.3), seems to have been unaddressed in terms of its implications for such a cycle. Where the NAD^+-dependent enzyme alone was found, as reported for the ascomycete *Chaetomium globosum* (Adomako *et al.*,

1972) and the basidiomycetes *Marasmius scorodonius* and *Mycena metata* (Ramstedt *et al.*, 1987), the cycle would be unable to regenerate NADPH as proposed. An alternative and extended model of the mannitol cycle was proposed in which the direct conversion of fructose to fructose 6-phosphate was intercalated with steps involving the conversion of fructose to sorbitol, followed by glucose, glucose 6-phosphate, and then fructose 6-phosphate (Figure 1.3B) (Jennings and Burke, 1990).

Improved methods in targeted gene inactivation, and the existence of strains of fungi which are amenable to such methods, have offered the means by which the existence of the mannitol cycle may be conclusively investigated. The mannitol 1-phosphate dehydrogenase gene was inactivated in *Aspergillus nidulans* (Ruijter *et al.*, 2003) and *Stagonospora nodorum* (Solomon *et al.*, 2005a) prior to the commencement of this study. While mannitol production was reduced in the mutant strains to 30% and 20% respectively of the wild types, it was evident that mannitol synthesis was possible in the absence of this enzyme. The first inactivation of a mannitol 2-dehydrogenase occurred in *S. nodorum* and was performed by the author as part of an Honours project (Waters, 2004). Although no mannitol 2-dehydrogenase activity could be detected in the mutant strain, it was otherwise phenotypically identical to the wild type, including the ability to grow on mannitol as a sole carbon source, offering the first evidence that the mannitol cycle does not exist as proposed, and suggesting a hitherto unknown means by which mannitol is catabolised. The possibility of creating a mutant harbouring both disruption constructs presented the opportunity of abolishing mannitol synthesis entirely and elucidating a role for mannitol metabolism in the infection process.

1.7 Summary and Aims

Stagonospora nodorum is a potent and economically significant necrotrophic pathogen of wheat. The availability of the genome sequence places an upper limit on the number and identity of genes. Targeted gene deletions have demonstrated the roles of several metabolites which are important during the three main stages of infection. Some of these have been suggested by the work of other studies, while others are the result of noted expression differences between EST libraries, alterations in gene expression and changes in metabolite abundance over the course of infection.

The aims of this project were to further probe the relationship between metabolism and infection in the *Stagonospora nodorum*-wheat pathosystem with particular reference to mannitol. A genetics approach was used initially to create a double mutant strain harbouring the disrupted mannitol dehydrogenase gene and mannitol-1-phosphate dehydrogenase gene. It was hypothesised that this would abolish the ability to synthesise or catabolise mannitol and would elucidate a role for this compound in infection. The strain was phenotypically characterised using standard *in vitro* and *in planta* growth assays and pathogenicity assays as established. Nuclear magnetic resonance techniques, including the use of ^{13}C-labelled substrates, were used to investigate changes in principal soluble metabolites between the wild type and mutant strains, and to gain an understanding of their pathways of metabolism. GC-MS metabolite profiling was used to observe changes in metabolic profile over the course of an infection by the wild type strain. It was a further aim of this study that it would suggest anti-fungal strategies by exposing weaknesses in the fungus' life-cycle.

CHAPTER 2 – GENERAL MATERIALS AND METHODS

2.1 Fungal and Bacterial Strains

Stagonospora nodorum strain SN15 was supplied by the Department of Agriculture, Western Australia, (now the Department of Agriculture and Food, Western Australia).

A *S. nodorum* mutant strain, *mpd1-1*, harbouring a disrupted mannitol 1-phosphate dehydrogenase gene was the generous gift of Dr. Peter Solomon (Solomon *et al.*, 2005a).

The *S. nodorum* mutant strains *mdh1-67*, *mdh1-71*, *mdh1-73*, *mdh1-78*, *mdh1-79* and *Mdh1-63e*, each harbouring a disrupted mannitol 2-dehydrogenase gene, were previously created by the author (Waters, 2004; Solomon *et al.*, 2007).

Escherichia coli DH10B (Grant *et al.*, 1990) (Invitrogen Corporation, Carlsbad, CA) containing the disruption construct pGPSH-Mpd8 was the generous gift of Dr. Peter Solomon. This construct comprises an insertionally mutagenised mannitol 1-phosphate dehydrogenase gene and confers resistance to hygromycin (Solomon *et al.*, 2005a).

2.2 Wheat Variety

The SN15-susceptible wheat cultivar, *Triticum aestivum* L. subsp. *aestivum* cv. Amery was used in pathogenicity assays and was supplied by the Department of

Agriculture, Western Australia, (now the Department of Agriculture and Food, Western Australia).

2.3 General Media

All media used within this study are listed in Table 2.1. All reagents used were of analytical grade. All water used was of milliQ quality and sterilised by autoclaving unless otherwise indicated. All autoclaving was carried out for 20 min at 121 °C and 100 kPa.

2.4 Growth of *Triticum aestivum* cv. Amery

Seeds were surface-sterilised for 5 min in sterilisation solution (1% bleach, 5% ethanol) followed by rinsing in sterile water. Sterile pots (125-130mm) were prepared by adding Expanded Perlite – Coarse (The Perlite & Vermiculite Factory, Jandakot) to the height of the drainage holes and filling the remainder of the pot to within approximately 1.5 cm of the top with vermiculite (The Perlite & Vermiculite Factory, Jandakot). Seeds were sown with 8 seeds per pot for a whole plant spray, or 50-60 seeds per pot for detached leaf assay – and covered with approximately 1 cm vermiculite. Pots were placed 8 to a sterile tray (Nally, NSW) and the tray filled with 2-3 cm of tap water. Seedlings were grown for 2 weeks in a growth chamber at 23 °C and a lighting regime of alternating 12 h light and 12 h dark. Lighting was provided by fluorescent tubes, 50% of which were GRO- LUX® F36W/Grow T8 (Sylvania, Germany) and the remainder Cool White L36 W/20 (Osram, Germany), with the two different types being mounted alternately.

Table 2.1: Media used in this study.

Medium	Ingredients
Benzimidazole Agar	1% w/v BBL™ agar (Becton, Dickinson & Co., USA) 100 mg.L^{-1} benzimidazole (ICN Biochemicals Inc, Aurora, USA)
Complete Supplement (for CzV8CS media)	20 g.L^{-1} Casamino acids (Becton, Dickinson & Co., USA) 20 g.L^{-1} Peptone (Becton, Dickinson & Co., USA) 20 g.L^{-1} Yeast extract (Becton, Dickinson & Co., USA) 3 g.L^{-1} Adenine (Sigma Chemical Co., St. Louis, USA) 0.02 g.L^{-1} Biotin (Sigma-Aldrich Inc.., St. Louis, USA) 0.02 g.L^{-1} Nicotinic acid (Sigma Chemical Co., St. Louis, USA) 0.02 g.L^{-1} p-aminobenzoic acid (Sigma Chemical Co., USA) 0.02 g.L^{-1} Pyridoxine (Sigma Chemical Co., St. Louis, USA) 0.02 g.L^{-1} Thiamine (Sigma Chemical Co., St. Louis, USA) Filter sterilise with 0.2 µm filter and store in fridge
CzV8CS Agar	45.4 g.L^{-1} Czapek Dox Agar (Oxoid) 10 g.L^{-1} BBL™ agar (Becton, Dickinson & Company) 3 g.L^{-1} $CaCO_3$ (Chem-Supply, Gillman, South Australia) 200 mL.L^{-1} filtered V8 Juice (Campbell's) pH 6.0 Autoclave 50 mL.L^{-1} Complete Supplement (see above) 50 µg.mL^{-1} phleomycin (Cayla, Toulouse) where required 200 µg.mL^{-1} hygromycin-B (Roche, Mannheim) where required
CzV8CS Liquid Culture Medium (for flask culture)	45.4 g.L^{-1} Czapek Dox Liquid Medium (Oxoid) 200 mL.L^{-1} centrifuged V8 juice (Campbell's) pH 6.0 Aliquot 95 mL per 250 mL flask Autoclave Add 5 mL.100 mL^{-1} complete supplement prior to use
CzV8-Proto Agar	45.4 g.L^{-1} Czapek Dox Agar (Oxoid) 10 g.L^{-1} BBL™ agar (Becton, Dickinson & Co., USA) 200 mL.L^{-1} centrifuged V8 Juice (Campbell's) 182.2 g.L^{-1} sorbitol (Univar International Ltd, Poole, England) pH 6.0 Autoclave and pour ~15 mL per plate

Continued on the following page

Table 2.1: (Continued)

Medium	Ingredients
CzV8-Proto Top Agar	45.4 $g.L^{-1}$ Czapek Dox Agar (Oxoid) 7.5 $g.L^{-1}$ BBL™ agar (Becton, Dickinson & Co., USA) 200 $mL.L^{-1}$ centrifuged V8 Juice (Campbell's) 182.2 $g.L^{-1}$ sorbitol (Univar International Ltd, Poole, England) pH 6.0 Autoclave
Luria-Bertani (LB) Broth	1% w/v bacto-peptone (Becton, Dickinson & Co., USA) 0.5% w/v yeast extract (Becton, Dickinson & Co., USA) 1% w/v NaCl (Univar, NSW) pH 7.0 Autoclave
Luria-Bertani (LB) Agar	As for LB broth but with 1.5% w/v agar added
Minimal Medium (MM) – Liquid (for flask culture)	30 $g.L^{-1}$ sucrose (Univar, NSW) 2 $g.L^{-1}$ $NaNO_3$ (Chem-Supply, Gillman, South Australia) 1 $g.L^{-1}$ K_2HPO_4 (Univar, NSW) 1 x trace stock solution (see below) pH 6.0 Autoclave
Minimal Medium minus Carbon (MM-C) - liquid	As for liquid MM but without sucrose
Minimal Medium (MM) - Solid	As for liquid MM but with the addition of 15 $g.L^{-1}$ BBL™ agar (Becton, Dickinson & Co., USA) 50 $\mu g.mL^{-1}$ phleomycin (Cayla, Toulouse) where required 200 $\mu g.mL^{-1}$ hygromycin-B (Roche, Mannheim) where required
Top Agarose	10 $g.L^{-1}$ Bacto™-Peptone (Becton, Dickinson & Co., USA) 5 $g.L^{-1}$ NaCl (Univar, NSW) 6 $g.L^{-1}$ agarose (Bio-Rad, Hercules, CA, USA) Autoclave
100x Trace Stock Solution (for Minimal Media)	50 $g.L^{-1}$ KCl (Rowe Scientific, Australia) 50 $g.L^{-1}$ $MgSO_4.7H_2O$ (Chem-Supply, Gillman, South Australia) 1 $g.L^{-1}$ $ZnSO_4.7H_2O$ (BDH Laboratory Supplies, Poole, England) 1 $g.L^{-1}$ $FeSO_4.7H_2O$ (Univar, NSW) 0.25 $g.L^{-1}$ $CuSO_4.5H_2O$ (Univar, NSW)

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Table 2.1: (Continued)

Medium	Ingredients
V8-Potato Dextrose Agar (V8-PDA)	150 mL L^{-1} V8 Juice (Campbell's) 10 $g.L^{-1}$ Potato Dextrose Agar 3 $g.L^{-1}$ $CaCO_3$ (Chem-Supply, Gillman, South Australia) 15 $g.L^{-1}$ Agar pH 6.0 Autoclave 50 $\mu g.mL^{-1}$ phleomycin (Cayla, Toulouse) where required 200 $\mu g.mL^{-1}$ hygromycin-B (Roche, Mannheim) where required

2.5 Growth of *Stagonospora nodorum*

2.5.1 Routine Maintenance and Culture

Both wild type and transformant strains of *S. nodorum* were routinely grown on solid media at 20 °C under alternating 12 h cycles of darkness and TL40W/05 (Philips, Holland) near-UV light. Liquid media were inoculated with 10^7-10^8 spores into 100 mL of medium, shaken continuously at 140 rpm for 3 days on a Certomat® R shaker (B. Braun, Melsungen, W. Germ.) at 20 °C in the dark. Strains of interest were preserved by 250-350 mg (wet weight) of mycelium/spores being resuspended in 20% glycerol, snap frozen in liquid nitrogen and stored at –80 °C.

2.5.2 Harvesting of Pycnidiospores

Pycnidiospores were harvested from sporulating plates, 2-3 weeks post-inoculation. The plate was flooded with 5 mL sterile water, and the surface scraped with a sterile pipette tip to remove aerial mycelium and ensure contact between the water and pycnidia. The plate was left for 10 min to allow the water to swell and burst pycnidia and release spores. A further 5 mL of sterile water was applied and the plate re-scraped. The spore solution which was aspirated with a sterile syringe and filtered through a sterile glass-wool-tipped 5 mL syringe. The solution was centrifuged for 5 min at 3500 g at room temperature and the pelleted spores resuspended in 1 mL sterile water. Approximately 20 μL of spore solution, diluted to 1:10 or 1:100 if required, were applied to a haemocytometer and spores counted under a light microscope to determine the concentration of spores. A concentration of 1 x 10^7 spores.mL^{-1} was sufficient for most procedures.

2.6 Growth of *Escherichia coli*

Escherichia coli cells were cultured overnight at 37 °C on solid or liquid LB or liquid SOC media. Media were supplemented with ampicillin (100 $\mu g.mL^{-1}$) as required. Liquid media were shaken at 225 rpm on a Certomat® R shaker (B. Braun, Melsungen, W. Germ.).

2.7 Nucleic Acid Extraction and Manipulation

2.7.1 Homogenisation of Fungal Mycelium/Pycnidiospores

Fungal mycelium was homogenised using a Retsch MM301 Mixer Mill (Retsch GmbH, Haan, Germany) as per the manufacturer's instructions. Approximately 250-350 mg (wet weight) mycelia/pycnidiospores were harvested from agar plates using a sterile scalpel blade. The mycelium was placed in a 2.0 mL safety-capped tube, frozen in liquid nitrogen and stored at –80 °C until required. Where pycnidiospores formed a significant portion of the tissue from which DNA was to be extracted, the sample was lyophilised overnight in a Savant FDC206 freeze drying chamber (Savant Scientific Instruments, Farmingdale, NY) attached to an Heto Maxi-Dry Lyo freeze dryer (Heto-Holten, Allerød, Denmark). A single 3 mm tungsten-carbide bead was placed in each tube prior to tissue lysis. Up to 5 tubes were placed in the Adaptor Set (with balances as appropriate) in liquid nitrogen to render the sample(s) metabolically inactive. Upon removal, the Adaptor Sets were knocked against the bench to ensure the tungsten-carbide beads were mobile. The Adaptor Sets were loaded into the Mixer Mill clamps and homogenised for 1 min at 30 Hz. This tissue homogenisation was repeated up to a further 2 times with samples being re-

frozen in liquid nitrogen and Adaptor Sets being knocked against the bench to free the beads each time before replacing in the Mixer Mill. Once the tissue resembled a fine powder 300 µL of Buffer RLT was added to each tube followed by vortexing. Tubes were centrifuged for 5 min at 6000 g at room temperature and lysate used immediately for genomic DNA extraction.

2.7.2 Genomic DNA Extraction from Lysed Fungal Mycelium/Pycnidiospores

Genomic DNA was extracted from homogenised tissue using a Qiagen BioSprint 15 (Thermo Electron Corporation, Finland) as per the manufacturer's instructions. The extracted DNA was stored at 4 °C until required.

2.7.3 Plasmid DNA Extraction

Plasmids were extracted using the Qiagen Midi-Prep Plasmid DNA Extraction Kit (Qiagen Pty Ltd, Clifton Hill, Vic, Australia) as per the manufacturer's instructions. Briefly, an overnight 50 mL bacterial culture was centrifuged at 6000 g for 15 min at 4 °C. The supernatant was discarded and the bacterial pellet resuspended in 4 mL chilled Buffer P1 to which RNase A had been added. This was followed by the addition of 4 mL Buffer P2. The tube was inverted gently 4-6 times to avoid shearing genomic DNA and incubated for 5 min at room temperature. A further 4 mL of chilled Buffer P3 was added, the tube inverted another 4-6 times and the entire volume poured into a Qiagen Cartridge and incubated for 10 min at room temperature. A Qiagen-Tip100 was equilibrated with 4 mL Buffer QBT after which it was removed to a fresh vessel. The Qiagen Cartridge was decapped and placed into the Qiagen-Tip,

the plunger inserted in the cartridge and the lysate filtered into the Tip. The Qiagen-Tip was then washed two times with 10 mL Buffer QC. DNA was then eluted into a new tube with 5 mL Buffer QF and 3.5 mL room temperature propan-2-ol was added. The volume was centrifuged at 5000 g for 60 min at 4 °C. The supernatant was discarded and the pellet was washed with a 2 mL 70% ethanol at 5000 g for 60 min at 4 °C. The supernatant was discarded and the pellet air-dried for 5-10 min. The pellet was then redissolved in 100 µL milliQ water.

2.7.4 Gel Electrophoresis of DNA

Agarose gels were prepared by dissolving 0.7% - 2.0% w/v Certified™ Molecular Biology Agarose (Bio-Rad, Hercules, CA) in 1x TAE Buffer (20 mM Tris, 10 mM glacial acetic acid, 1 mM EDTA). DNA gel electrophoresis was performed in a horizontal DNA Sub Cell™, Wide Mini-Sub® Cell GT or Mini-Sub® Cell GT (Bio-Rad, Hercules, CA) electrophoresis tank containing an agarose gel in 1x TAE Buffer. DNA samples were mixed with 1x Blue/Orange Loading Dye (Promega, Madison, WI) prior to loading into the gel. A lane containing 1 kb DNA Ladder (Promega, Madison, WI) was loaded to enable the determination of the approximate molecular weight of DNA bands. Electrophoresis tanks were attached to a Power Pac 300 (Bio-Rad, Hercules, CA). Gels were electrophoresed until the dye front had progressed ¾ to 4/5 the length of the gel. The voltage used for electrophoresis varied with the size of the gel, the sizes of the expected DNA bands, and the degree of separation/definition of bands required. After completion of the run, gels were stained with 0.5 $\mu g.mL^{-1}$ ethidium bromide for 30 min and visualised under UV light in a Bio-Rad Gel-Doc 1000 running Bio-Rad Molecular Analyst™ Version 1.4.

2.7.5 Determination of DNA Concentration

Prior to measurement, tubes containing extracted DNA were spun down in a benchtop microcentrifuge at 6000 g for 1 min at room temperature to remove interference in measurement by any MagAttract® Suspension G carried over from the extraction process. DNA concentration was determined by a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) running NanoDrop ND-1000 Version 3.1.2 (Coleman Technologies Inc., USA) as per the manufacturer's instructions. Briefly, 1-2 µL of DNA extract was pipetted onto the lower measurement pedestal, the sampling arm lowered into position, and the DNA concentration reading was taken. The upper and lower measurement pedestals were cleaned prior to any measurement, and the NanoDrop was blanked with the same solvent used to store the extracted DNA.

2.7.6 Restriction Endonuclease Digestion of DNA

Restriction digestion of DNA was performed with enzymes purchased from Promega (Madison, WI) or Fermentas Life Sciences (Hanover, MD) as per the manufacturer's instructions. Each reaction contained 10x appropriate enzyme buffer, 10x BSA, excess enzyme(s) and DNA, with sterile water used to make up the final reaction volume. Reactions were performed at the temperature specified for the enzyme(s) used and incubation times ranged from 1.5 h to overnight. Reaction volumes ranged from 15 µL (typically for analysis of plasmid minipreps) to 1 mL (for linearisation of disruption constructs).

2.7.7 Purification of Linearised Plasmid DNA

Plasmid DNA was purified using a QIAquick PCR Purification Kit (QIAGEN Pty Ltd, Clifton Hill, Vic, Australia) as per the manufacturer's instructions. Briefly, 5 volumes of Buffer PB were added to 1 volume of PCR sample. The whole volume was placed in a QIAquick spin column which was inserted into a 2 mL collection tube and DNA bound by centrifugation at 17900 g for 30 sec at room temperature. The eluent was discarded, 750 µL Wash Solution (Buffer PE) added and the tube centrifuged at 17900 g for 30 sec at room temperature. The supernatant was discarded and the tube again centrifuged at 17900 g for 60 sec at room temperature. The spin column was removed to a clean 1.5 mL eppendorf tube and 10-30 µL STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) was added to the membrane and the tube allowed to stand for 1 min. The tube was centrifuged at 17900 g for 60 sec at room temperature and the purified DNA stored at -20 °C until required.

2.7.8 DNA Amplification by Polymerase Chain Reaction

PCR amplification of DNA was carried out in 25 µL reactions containing 2.5 µL 10x Buffer (Promega, Madison, WI), 1.0 µL 10 mM dNTPs (Promega, Madison, WI), 0.5 µL 10 µM primer per primer used in the reaction, 0.2 µL *Taq* polymerase (Promega, Madison, WI), a variable amount of DNA template and made up to 25 µL with sterile water. Genomic DNA of transformants was routinely screened with actin control primers actinF (5'-CTGCTTTGAGATCCACAT-3') and actinR (5'-GTCACCACTTTCAACTCC-3') (Solomon *et al.*, 2003) to confirm the presence of DNA in DNA extractions. These primers amplified a band of approximately 300 bp.

PCRs were performed in a GeneAmp® PCR System 2400, 2700 or 9700 (Perkin Elmer Applied Biosystems) thermocycler or a PTC-240 DNA Engine Tetrad® 2 Peltier Thermal Cycler (M J Research Inc., Waltham, Mass, USA; Bio-Rad Laboratories Inc., Hercules, CA, USA) depending on the number of samples. Thermocycler conditions consisted of an initial denaturing step of 2 min at 96 °C, 40 repeats of 10 sec at 96 °C, 20 sec at a variable annealing temperature, and 30 sec at 72 °C, a final extension step of 5 min at 72 °C, and a hold at 14 °C until ready for gel electrophoresis.

2.8 Gas Chromatography – Mass Spectrometry

2.8.1 Extraction of Polar Metabolites

Biological material (fungal and/or plant) was harvested and snap frozen in liquid nitrogen. Each sample was ground in liquid nitrogen in a pre-chilled sterile mortar and pestle. Each ground sample was divided into four aliquots. Three aliquots consisted of 10-100 mg sample and were placed into three pre-weighed eppendorf tubes. The tubes were re-weighed to determine the weight of the sample in each tube. These aliquots were later assayed for DNA concentration in order to normalise the GC-MS sample data. The fourth aliquot for each sample consisted of 0.5-2.0 mg sample and was transferred to a pre-weighed 2.0 mL eppendorf containing 1 mL methanol and vortexed briefly to render the sample biologically inactive. This transfer was performed as quickly as possible to minimise evaporation of methanol. The tube was re-weighed to determine the weight of the sample. Each sample had 50 µL 0.2 $mg.mL^{-1}$ ribitol (Sigma-Aldrich Inc., St Louis, MO, USA) added to act as an internal

standard, and 100 µL non-autoclaved milliQ water. Samples were inverted 5-6 times, the tubes capped with safety caps, and incubated in an Eppedorf Thermomix (Hamburg, Germany) shaking/heating block at 70 °C for 15 min with shaking at 1000 rpm. Tubes were centrifuged in an Eppendorf Centrifuge (Model 5417C, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 14,000 g for 3 min and the supernatant transferred to a fresh tube. The pellet was resuspended in 500 µL water plus 375 µL chloroform, vortexed, and incubated at 37 °C for 5 min with shaking at 1000 rpm. The tubes were centrifuged at 14,000 g for 3 min and the upper polar fraction transferred to the fresh tube containing the supernatant from the first incubation. Samples were then placed in a Savant SpeedVac® vacuum concentration chamber (Savant Scientific Instruments, Farmingdale, NY) attached to a Savant RT400 Refrigerated Condensation Trap (Savant Scientific Instruments, Farmingdale, NY) and evaporated to dryness overnight. Dried samples were stored at -80 °C for short periods until required for derivatisation.

2.8.2 Derivatisation of Polar Metabolite Extracts

Methoximation of the carbonyl groups of the dried polar extracts was achieved by the addition of 50 µL freshly prepared methoxylamine hydrochloride (Aldrich, St Louis, MO, USA) (20 mg/mL^{-1} in pyridine (Univar, Seven Hills, NSW)) per sample, followed by were incubation for 90 minutes at 30 °C with shaking at 1200 rpm. Trimethylsilyl (TMS) esters were created by the addition of 80 µL N-methyl-N-(trimethyl-silyl) trifluoroacetamide (MSTFA) (Sigma-Aldrich Inc., St Louis, MO, USA) to the sample and incubation for 30 min at 37 °C with shaking at 1200 rpm.

Finally, 100 µL of the derivatised sample was transferred to an 11 mm crimp 2 mL clear glass GC-MS vial containing a glass insert (Alltech, Deerfield, Il, USA), 10 µL alkane mix added, and sealed with an 11 mm aluminium seal with rubber faced liner (Alltech, Baulkham Hills, NSW). The alkane mix consisted of 62.5 $\mu g.mL^{-1}$ each of C10, C12, C15, C19, C22, C26, C32 and C36 dissolved in hexane. Samples were stored at room temperature for at least 2 hours prior to GC-MS analysis. Where the sample contained obvious suspended sediments, these were pelleted by centrifuging at 14,000 g for 1 min prior to loading the sample into the vial.

2.8.3 Gas Chromatography – Mass Spectrometry

Samples were injected as 1 µL derivatised metabolites in a 1:20 sample:methanol split ratio. The GC-MS system comprised an Agilent 7680 autosampler, Agilent 6890 gas chromatograph, and Agilent 5973N quadropole mass spectrometer (all Agilent Technologies, Palo Alto, CA, USA). The system was autotuned with perfluorotributylamine as per the manufacturer's instructions. A 30-m HP-50+ column with a 250 µm internal diameter and 0.25 µm film thickness was used for gas chromatography (J&W Scientific, Folsom, CA, USA).

The operational temperatures were 230 °C (injection), 300 °C (interface) and 230 °C (ion source). The carrier gas used was helium with a flow rate retention time locked to elute mannitol-TMS at 24.51 min. The GC oven temperature gradient consisted of an initial temperature of 70 °C for 5 min, followed by ramping at 5 $°C.min^{-1}$ to a final temperature of 300 °C held for 3 min.

Mass spectra and chromatograms were analysed using AnalyzerPro V2.2 (SpectralWorks Ltd, Runcorn, UK). Peak identification was based on comparison of unknowns to the ACNFP GC-MS TMS Library, the NIST/EPA/NIH Mass Spectral Library (NIST, Gaithersburg, MD, USA) or the Golm Metabolome Database (Kopka *et al.*, 2005) using NIST Mass Spectral Search Program 2.0 (NIST, Gaithersburg, MD, USA).

2.8.4 Data Normalisation

All GC-MS samples contained an internal standard consisting of 50 µL 0.2 $mg.mL^{-1}$ ribitol which was added during the polar metabolite extraction step. All metabolite peak areas were divided by the peak area for ribitol, and then divided by the wet weight of the sample to enable qualitative comparison between samples.

2.9 Software

Sequencing and homologous recombinant screening primer design, CLUSTAL W alignments and Boxshading were performed using ANGIS Biomanager (ANGIS, 2004). Restriction sites of nucleotide sequences were analysed using NEBcutter V2.0 (Vincze *et al.*, 2003). Sequence editing, manipulation and alignment for base corrections and contig formation were performed using SEQtools version 8.2.067 (Rasmussen, 2004). Comparisons of nucleotide and amino acid sequences against databases were performed with the BLAST suite of programs (Altschul *et al.*,

1997). Searches for protein motifs were performed using FingerPRINTScan (EBI, 2004a) and PPSearch (EBI, 2004b).

2.10 Statistical Analysis

Statistical analysis of data was performed with JMP IN V5.1 (SAS Institute Inc, Cary, NC, USA) software using the Tukey-Kramer Honestly Significant Difference (HSD) test with an alpha level of 0.05.

CHAPTER 3 - CONSTRUCTION AND CHARACTERISATION OF A STRAIN OF *Stagonospora nodorum* HARBOURING DISRUPTED GENES FOR MANNITOL 2-DEHYDROGENASE (*mdh1*) AND MANNITOL 1-PHOSPHATE 5-DEHYDROGENASE (*mpd1*).

3.1 INTRODUCTION

3.1.1 Nomenclature, Class and Structure of D-Mannitol

D-Mannitol belongs to the class of chemical compounds known as polyols or polyhydric alcohols. These compounds are derived from precursor aldose or ketose monosaccharides via the reduction of their carbonyl group to a hydroxyl group (Brimacombe and Webber, 1972). D-Mannitol is a 6-carbon acyclic polyol which is formed by the reduction of D-mannose or D-fructose, and derives its name from the former of these two compounds. Since L-mannitol is not found in nature, the D-form is commonly referred to as mannitol and this convention will be observed hereon. The configuration of the hydroxyl groups on the carbon skeleton renders mannitol a symmetric molecule (Figure 3.1).

3.1.2 Taxonomic Distribution

Mannitol, known previously as mannite or sugar of manna (Dunglison, 1856) was first isolated from manna exuded from plants in 1806 by Proust (Ihde, 1984) and was found to form a principal component of the basidiomycete *Agaricus volvaceus* in 1811 by Braconnot (Thomson, 1817; Gobley, 1856). One of the first systematic reviews of the distribution of polyols concluded that mannitol was the most abundant polyol in fungi and, with few exceptions, was present in all fungi studied (Lewis and Smith, 1967). Subsequent reviews have shown this to be true for the fungi of the phyla basidiomycota and ascomycota (with the exception of the yeasts and fission yeasts of the subphyla saccharomycotina and taphrinomycotina), while mannitol was

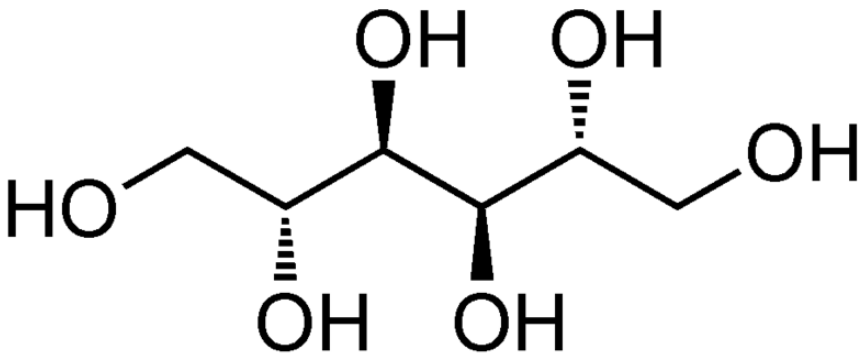

Figure 3.1: The structure of D-mannitol (Fischer projection)

not found among the polyols of the phylum blastocladiomycota or the taxonomically unclassifiable imperfect fungi assigned to the group *fungi incertae sedis* (Rast and Pfyffer, 1989). Mannitol is reported as comprising up to 50% of the dry weight of the fruiting bodies and 20% of the dry weight of mycelium of *Agaricus bisporus* (Rast, 1965) and 10-15% of the dry weight of the conidiospores of *Aspergillus niger* (Ruijter *et al.*, 2003). Mannitol is considered to be ubiquitous in lichens, found in the majority of algae, and in the case of angiosperms it was the most widely distributed of the polyols, being found in over 50 families (Lewis and Smith, 1967).

While only infrequently reported from animals, mannitol has been found to occur naturally in insects (Wang *et al.*, 2006) and humans (Servo *et al.*, 1977a; Servo *et al.*, 1977b; Laker *et al.*, 1982). Mannitol is also found in bacteria (Coyne and Raistrick, 1932; Edwards *et al.*, 1981; Wisselink *et al.*, 2002) and apicomplexa (Schmatz *et al.*, 1989). It is considered to be the most abundantly occurring polyol in nature (Wisselink *et al.*, 2002). The near ubiquitous presence of mannitol as the major soluble metabolite in phytopathogenic fungi has previously been recognised as providing a potential antifungal target (Pfyffer *et al.*, 1990).

3.1.3 Mannitol Metabolic Pathways in *Stagonospora nodorum*

There are a number of enzymes which have previously been reported as being involved in mannitol metabolism in fungi (Table 1.3). Some of these have been purported to occur as part of an enzymatic cycle (Figure 1.3). Two of the key enzymes in this proposed cycle have been previously inactivated by targeted gene deletion in *S. nodorum*. An EST library of *S. nodorum* genes expressed when grown

on wheat cell walls, produced a cDNA which encoded a putative mannitol 1-phosphate dehydrogenase (*Mpd1)* (EC 1.1.1.17) (Solomon *et al.,* 2005a). The gene was disrupted by insertional mutagenesis using the knockout vector pGPSH-Mpd8 (Figure 3.2) and mutants lacked all detectable mannitol 1-phosphate dehydrogenase activity in both directions. *In vitro* cultured mycelium contained approximately 20% of the wild type levels of mannitol, and also contained less arabitol and more trehalose than the wild type as shown by NMR (Solomon *et al.*, 2005a). Similarly, the conidia of the *mpdA* mutants in *A. niger* were found to contain 30% of the mannitol content of wild type conidia (Ruijter *et al.,* 2003). These results suggested that the catabolic half of the mannitol cycle must be capable of operating in reverse in order to synthesise mannitol. A fructose 6-phosphate phosphatase activity, which would be required in order for this to happen, has previously been reported in fungi (Morton *et al.*, 1985b; Kulkarni, 1990; Chakraborty *et al.*, 2004).

Using a reverse genetics approach, the gene encoding a putative mannitol dehydrogenase gene (*Mdh1*) was cloned by degenerate PCR and insertionally mutagenised using the knock-out vector pGPSP-Mdh1 (Figure 3.3) (Waters, 2004). It was hypothesised, based on the mannitol cycle theory, that mutants in which this gene was inactivated would be unable to catabolise mannitol and that this would elucidate a clear role for mannitol in pathogenicity. The *mdh1* mutants were found to lack all mannitol dehydrogenase activity, yet were phenotypically identical to the wild type in all other respects, including the ability to grow on mannitol as a sole carbon source, and the ability to cause disease and sporulate *in planta*. The clear implication from the *mdh1* mutants was that the mannitol cycle could not exist as proposed, since catabolism of mannitol would require the reversal of the dephosphorylation step from

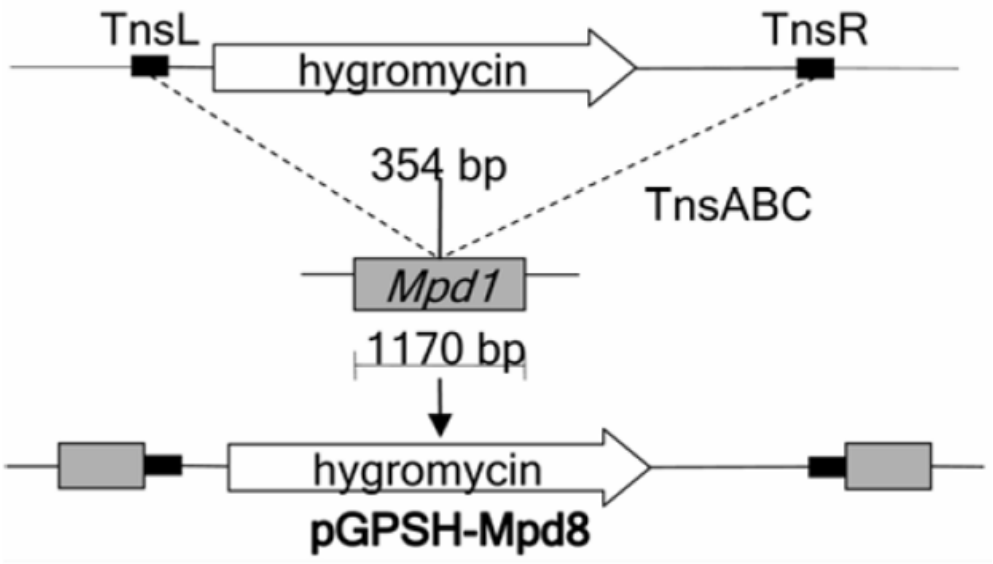

Figure 3.2: Diagram outlining the construction of the knockout vector pGPSH-Mpd8 (taken from Solomon *et al*. (2005)). Note the transposon inserted at 354 bp downstream of the predicted start codon, as determined by sequencing with primers homologous to the transposon termini TnsL and TnsR.

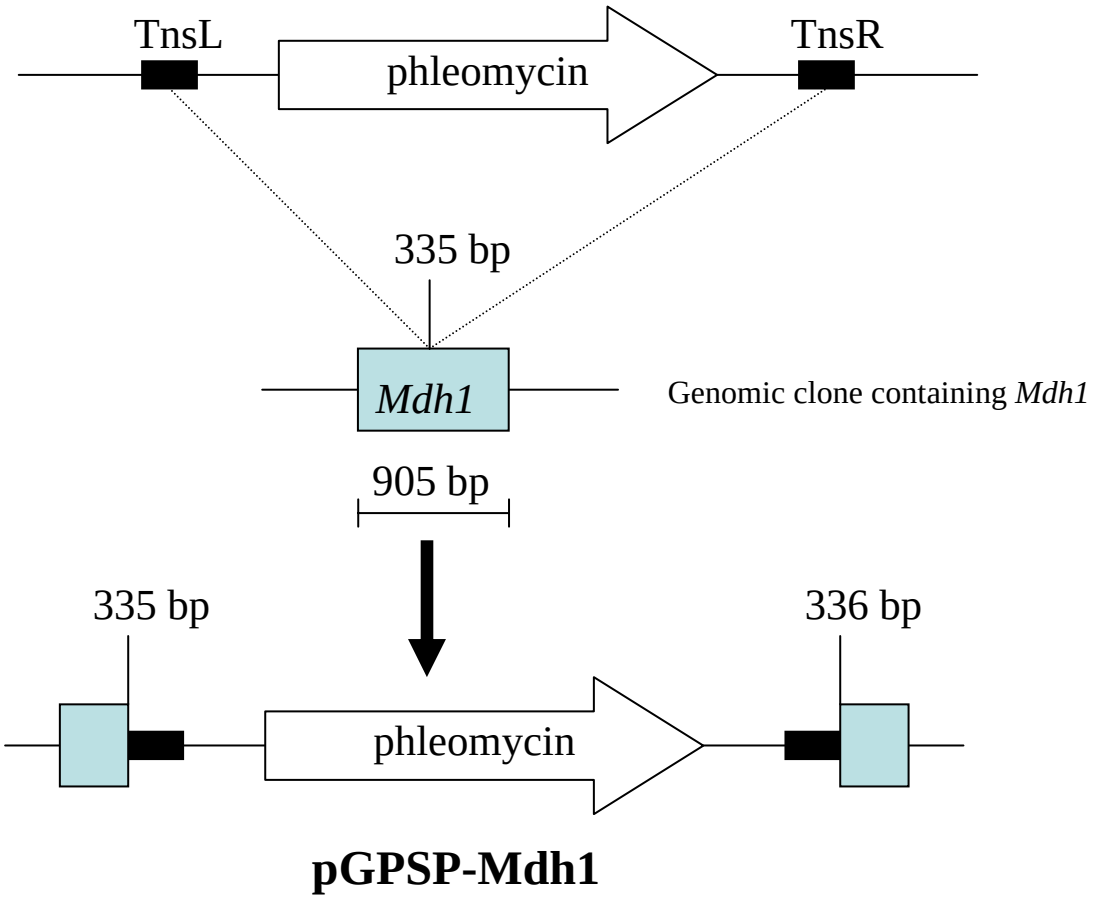

Figure 3.3: Diagram outlining the construction of the knockout vector pGPSP-Mdh1. Note the transposon inserted at 335 bp downstream of the predicted start codon, as determined by sequencing with primers homologous to the transposon termini TnsL and TnsR.

mannitol 1-phosphate to mannitol, catalysed by mannitol-1-phosphate phosphatase. This would be most simply achieved by the presence of a mannitol kinase enzyme. However, apart from reports of a mannitol kinase in *Microsporum gypseum* (Leighton *et al.*, 1970) and *Absidia glauca* (Ueng *et al.*, 1976; Ueng and McGuinness, 1977), no such activity has previously been found in fungi (Lones and Peacock, 1964; Lee, 1967b; Strandberg, 1969; Adomako *et al.*, 1972), including *S. nodorum* (P. Solomon, pers. comm.).

The creation of a double transformant strain in which both the *Mdh1* and *Mpd1* genes were disrupted was necessary for the further elucidation of the metabolism of this compound. If these represented the only two mannitol metabolic pathways, then it would be possible to abolish mannitol synthesis altogether, and also produce a strain which would be unable to utilise mannitol as a sole carbon source. Furthermore, it would be possible to finally demonstrate the role(s) of mannitol. In the event that mannitol was still metabolised, there would be clear evidence for an alternative pathway of mannitol biosynthesis. The first part of the current project was, therefore, to create and characterise a double mutant strain which harboured both inactivated gene constructs.

3.2 MATERIALS AND METHODS

3.2.1 Fungal Transformation

3.2.1.1 Preparation of Protoplasts

The *S. nodorum* strain *mdh1-71*, harbouring a disrupted mannitol 2-dehydrogenase gene construct, pGPSP-Mdh1 (Figure 3.3 above), conferring phleomycin resistance (Waters, 2004), was selected as the background for the double mutant strain. Mycelium for preparation of protoplasts was obtained by inoculating 100 mL CzV8CS liquid medium with not less than 10^8 *mdh1-71* pycnidiospores and incubating for approximately 20 h in the dark at 20 ºC with shaking at 140 rpm. The culture was centrifuged at 3000 g for 10 min at room temperature. The mycelium pellets were resuspended in 50 mL 0.02 μm filter-sterilised wash solution (0.6 M $MgSO_4$) and washed at 3000 g for 5 min at room temperature. The pellet was resuspended and well mixed in 25 mL 0.02 μm filter-sterilised Glucanex digestion solution (1.2 M $MgSO_4$, 10 mM NaH_2PO_4, 15 $mg.mL^{-1}$ Glucanex (Novo Nordisk Ferment Ltd., Dittingen, Switzerland), pH 5.8) and incubated for 2 h at 28 ºC without agitation in a pre-warmed sterile glass petri dish to digest cell walls. The protoplasts were transferred to a sterile 50 mL Falcon tube and the solution overlaid with 5 mL Protoplast Overlay Solution (0.6 M sorbitol, 10 mM Tris-HCl pH 7.5) and centrifuged at 3500 g for 15 min at room temperature. Up to 1 mL of protoplasts were removed from the interface layer and transferred to a 2 mL eppendorf tube. An equal volume of 1 M Sorbitol Solution (1 M sorbitol, 10 mM Tris-HCl pH 7.5) was added, the contents gently mixed by pipetting and centrifuged at 1500 g for 5 min at room

temperature. The pelleted protoplasts were resuspended in 2 mL STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5) and washed at 1500 g for 5 min at room temperature. The pellet was resuspended in 0.5 mL STC buffer and kept on ice until ready for transformation. A 1:500 dilution was prepared in STC buffer and the protoplasts counted using an haemocytometer under an Olympus CX41RF (Olympus Optical Co. Ltd., Philippines) light microscope. Ideally, a concentration of no less than 5 x 10^8 protoplasts.mL^{-1} was required for transformation.

3.2.1.2 Transformation of Protoplasts

Transformation of protoplasts was essentially as described in Cooley *et al.* (1988). *Escherichia coli* cells containing the pGPSH-Mpd8 (Figure 3.2) plasmid were cultured overnight and plasmid DNA extracted. Sufficient DNA for two transformations was linearised by digesting with the restriction endonuclease Apa1 (Promega, Madison, WI) for 3 h at 37 °C. The linearised DNA was purified using a QIAquick PCR Purification Kit (QIAGEN, Clifton Hill, Vic, Australia) as described above and resuspended in 10-30 μL STC buffer (1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5). The concentration of DNA in the final solution was assayed spectrophotometrically and 1 μL of the resuspended DNA was run on a 0.7% agarose gel to ensure the plasmid was completely linearised and that only a single band was present. Bands were visualised with a Bio-Rad Gel Doc 1000 using Perkin-Elmer™ UV Winlab Version 2.85.04. The linearised construct was stored at –20 ºC until required.

Transformations were carried out in duplicate for each experiment. For each transformation, approximately 10^8 protoplasts were transferred to a 2 mL eppendorf tube, not less than 7.5 μg linearised DNA added, and the volume made up to 125 μL with STC buffer if required. The contents were gently mixed with a P1000 pipette and 1 mL tip and incubated at room temperature for 15 min. PEG-mediated transformation was conducted by adding 200 μL 60% PEG solution (60% PEG-4000, 10 mM $CaCl_2$, 10 Tris pH 7.5) to the DNA with mixing by inversion and incubation for 30 sec at room temperature. A further 200 μL 60% PEG solution was added, mixed by inversion and incubated for 30 sec. Finally 800 μL 60% PEG solution was added, mixed by inversion and incubated at room temperature for 15 min.

For each transformation experiment 5 mL 50 ºC CzV8-Proto Top Agar was added to each of 4 pre-warmed (50 ºC) 10 mL sterile Falcon tubes, 310 μL transformed protoplasts were added to each tube, the contents mixed and poured onto a 15 mL CzV8-Proto Agar plate. For controls, 100 μL untransformed protoplasts were added to each of an additional 2 pre-warmed (50 ºC) 10 mL sterile Falcon tubes containing 5 mL 50 ºC CzV8-Proto Top Agar, mixed and poured onto 15 mL CzV8-Proto Agar plates. All plates were wrapped in clingfilm, covered with aluminium foil and incubated for 40 h at 20 ºC. After this incubation period 5 mL 50 ºC CzV8-Top Agar containing 5.0 mg hygromycin per mL agar (i.e. 200 μg hygromycin per mL agar on the plate) was overlaid on each transformed protoplast plate, and on one of the untransformed protoplast plates. Plates were all rewrapped in clingfilm, covered in foil and incubated at 20 ºC for 1-3 weeks, with transformant colonies being sub-cultured onto fresh CzV8CS + hygromycin plates as they appeared (3 to a plate). For

this particular transformation the CzV8-Proto Top Agar and CzV8-Proto Agar were augmented with 1 mM mannitol.

3.2.1.3 Screening of Transformants

Approximately 2 weeks after sub-culturing, 200-250 mg (wet weight) of mycelium were harvested from transformant colonies into a 1.5 mL eppendorf tube, snap frozen in liquid nitrogen and stored at –80 ºC until DNA extraction was performed as described (Section 2.7). Preliminary screening for homologous recombinants was conducted by PCR amplification using the primers mpdkoF (5'-GAGTTCACCATCGACCACT-3') and mpdkoR (5'-TACTGCTTCTTCGCCTGG-3') predicted to amplify a band of 493 bp within a non-disrupted mannitol 1-phosphate dehydrogenase gene. Presence of an amplified band indicated a putative ectopic insertion and absence of an amplified band indicated a putative homologous recombinant. PCR with actin primers was required in the latter instance to demonstrate that there was genomic DNA in the sample. Putative homologous recombinants were also screened with the primers mdhKOPcrF (5'-ACCGAGCTCA AGGACCTCT-3') and mdhKOPcrR (5'-AACGAGGGAGCCAGTCTTG-3') predicted to amplify a band of 461 bp within a non-disrupted mannitol 2-dehydrogenase gene. This was a precautionary step to confirm the stable integration of the pGPSP-Mdh1 gene disruption construct. The annealing temperature for all primer pairs used was 57 ºC.

3.2.1.4 Sub-Culturing of Transformant Colonies

Selected strains of putative ectopics and homologous recombinants identified by PCR were subcultured in order to ensure a homogeneous culture for further analysis. Approximately 100-150 mg (wet weight) mycelium/pycnidiospores were harvested by scraping a sterile scalpel across the surface of the colony, transferred to a 1.5 mL eppendorf tube containing 1 mL sterile water and vortexed to resuspend the fungal tissue. A 1:100 dilution was made with sterile water and 100 µL spread on a fresh CzV8CS agar plate containing appropriate fungicides. Plates were wrapped in clingfilm and incubated at 20 ºC under lighting conditions of alternating 12 h darkness and 12 h TL40W/05 (Philips, Holland) near-UV light until individual colonies could be seen. A discrete single colony was transferred to a fresh CzV8CS agar plate containing appropriate fungicides, wrapped and incubated as previously. The resulting colony was used as a source of inoculum for glycerol stocks from which the strain was regenerated for experimental manipulation and Southern analysis to confirm single integration of the disruption construct

3.2.2 Southern Hybridisation

3.2.2.1 PCR Amplification of DNA Probes

DNA probes for Southern analysis were amplified using the primer pairs mdhSOUTHF (5'-GTCGATGTCTTCATTGCCA-3') and mdhSOUTHR (5'-GAAGTAGACGTAAGCGCCCT-3'), predicted to amplify a band of 393 bp, and mpdSOUTHF (5'-AGTTCCTTCACAACTCTGGCT-3') and mpdSOUTHR (5'-

GATGAAGCCCCTCCATGT-3'), predicted to amplify a band of 311 bp. PCR amplification was carried out in 50 µL reactions containing 5.0 µL 10x Buffer (Promega, Madison, WI), 1.0 µL 10 mM dNTPs (Promega, Madison, WI), 2.5 µL 10 µM primer per primer used in the reaction, 0.5 µL *Taq* polymerase (Promega, Madison, WI), a variable amount of DNA template and made up to 50 µL with sterile water. PCRs were performed in a GeneAmp® PCR System 2400, 2700 or 9700 (Perkin Elmer Applied Biosystems) thermocycler. Thermocycler conditions consisted of an initial denaturing step of 2 min at 94 °C, 35 repeats of 20 sec denaturing at 94 °C, 20 sec annealing at 57 °C, and 40 sec extending at 72 °C, a final extension step of 5 min at 72 °C, and a hold at 14 °C until ready for gel electrophoresis.

3.2.2.2 DIG-Labelling of DNA Probes

This procedure was performed using the DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Mannheim) as per the manufacturer's instructions. A volume containing 1 µg of PCR-amplified template DNA was made up to 16 µL with sterile water, denatured by heating in a thermocycler at 99.9 °C for 10 min and immediately chilled on ice. DIG-High Prime was thoroughly mixed by pipette and 4 µL was added to the denatured DNA and centrifuged briefly, followed by overnight incubation in a thermocycler at 37 °C. The reaction was stopped by heating in a thermocycler at 65 °C for 10 min and the DIG-labelled probe stored at –20 °C until required.

3.2.2.3 Genomic DNA Digestion and Electrophoresis

Genomic DNA was extracted from the strains of interest (Section 2.7) and digested in a 200 µL reaction containing 20 µg gDNA, 20 µL 10x buffer, 7 µL restriction endonuclease and made up to the final volume with milliQ water. Strains being analysed with the mdhSOUTH probe were digested with HindIII (Promega, Madison, WI, USA) and strains being analysed with the mpdSOUTH probe were digested with Pst1 (Promega, Madison, WI, USA). Digests were incubated overnight at the manufacturer's recommended temperature of 37 °C. The digested DNA was concentrated in a Savant SpeedVac® vacuum concentration chamber (Savant Scientific Instruments, Farmingdale, NY) attached to a Savant RT400 Refrigerated Condensation Trap (Savant Scientific Instruments, Farmingdale, NY) for approximately 1 hour to produce a final volume of 25 µL. The concentrated DNA was mixed with 1x Blue/Orange Loading Dye (Promega, Madison, WI, USA) and loaded into the wells of a 250 mL 0.9% agarose gel and electrophoresed overnight at 30 V.

3.2.2.4 Southern Blot

Agarose gels for Southern blotting were electrophoresed until the dye front had progressed 2/3 to 4/5 the length of the gel. Transfer of electrophoresed DNA from the agarose gel to a membrane was performed on a VacuGene XL (Pharmacia Biotech) vacuum tray attached to a VacuGene Pump (Pharmacia Biotech) using a clean nylon mask which was approximately 5 mm smaller at each margin than the agarose gel. An Hybond™ N+ positively charged nylon transfer membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK) was cut to the same

dimensions as the gel, and the apparatus assembled with the membrane beneath and overlapping the nylon mask. The gel was placed such that it overlapped the mask and the vacuum pump turned on and set to 50 mbar to ensure an intact seal. The gel was serially covered with 50 mL Depurination Solution (0.2 M HCl), 50 mL Denaturing Solution (1.5 M NaCl, 0.5 M NaOH) and 50 mL Neutralising Solution (1.5 M NaCl, 0.5 M Tris-HCl pH 8.0). Each solution was left *in situ* for 20 min following which it was removed by pipette. Finally 20x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) was used to flood the tray to a depth 2x the height of the gel. After 1 h the wells were marked on the membrane with an HB pencil and the SSC was poured off. The top left hand corner of the membrane was cut off and the membrane placed on Whatman Chromatography Paper 3mm Chr. to dry. The membrane was then UV cross-linked in a Gene-Linker (Bio-Rad, Hercules, CA) at 150 mJoule and stored at 4 °C until required.

3.2.2.5 Hybridisation and Immunological Detection

Hybridisation and immunological detection was carried out using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche, Mannheim) as per the manufacturer's instructions, in an Hybridization Oven/Shaker (Amersham Pharmacia Biotech, Buckinghamshire, UK). Hybridisation membranes were pre-hybridised in an hybridisation tube with 20 mL DIG Easy Hyb and incubated for 30 min at 42 °C with constant rotation. The solution was replaced with the appropriate denatured probe and hybridised overnight at 42 °C. Excess probe was removed by stringency washes of 2 x 5 min in 100 mL 2x SSC, 0.1% SDS at 42 °C under constant agitation, and 2 x 15 min in 100 mL 0.5 SSC, 0.1% SDS at 68 °C under constant

agitation. Immunological Detection commenced with washing the membrane for 1-5 min in Washing Buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5 (20 °C), 0.3% v/v Tween 20), 30 min incubation in 30 mL 1x Blocking Solution, and 30 min incubation in 20 mL Antibody Solution. Membranes were then washed for 2 x 15 min in 100 mL Washing Buffer, equilibrated for 5 min in 20 mL Detection Buffer, and placed DNA-side up on an hybridisation bag. To the membrane was applied 1 mL CDP-Star, the hybridisation bag was sealed and excess fluid expelled, and the membrane was incubated for 5 min at room temperature. The membrane was exposed to Lumi-Film Chemiluminescent Detection Film (Roche Diagnostics, Indianapolis) for 5 min to overnight as required, and the film developed in a Fuji X-ray film processor FPM 3000.

3.2.3 *In vitro* Growth Assays

3.2.3.1 Growth on Solid Media

Selected strains were inoculated onto minimal medium, CZV8CS, and V8-PDA agar plates (with phleomycin and/or hygromycin added as appropriate). The inoculum consisted of 20 µL of a 10^6 spores.mL^{-1} spore suspension. Each strain was prepared in triplicate and plates were wrapped in clingfilm and incubated at 20 °C under a lighting regime of alternating 12 h darkness and 12 h TL40W/05 (Philips, Holland) near-UV light. Measurements of colony diameter along a fixed line through the centre of the colony were taken with a caliper at regular intervals and observations made with regard to colony morphology.

3.2.3.2 Ability to Grow on Selected Carbon Sources

Selected strains were assayed for their ability to grow on selected carbon substrates. All assays were performed in triplicate for each strain using a 96 well microtitre plate with 180 µL medium and 20 µL of a 10^6 spores.mL^{-1} spore suspension per well. Media consisted of liquid minimal medium as described (Table 2.1) but with carbon being replaced with glucose, fructose, sucrose, trehalose or mannitol; or liquid minimal media with no carbon or nitrogen, supplemented with either casamino acids or casamino acids plus glucose. In all cases the final concentration of the compound in the medium was 25 mM except for the casamino acids, where a final concentration of 1 g.L^{-1} was used. A control treatment consisting of minimal medium with no carbon source was also prepared. An initial reading of the plate at A_{595} was taken using a Beckman Coulter® DTX 880 Multimode Detector (Wals, Austria) running Multimode Detector Software Version 2.0.0.12 (Beckman Coulter Inc.). The plate was covered and wrapped in clingfilm and incubated in the dark at 20 °C. After 1 week, a second reading at A_{595} was taken and fungal growth was measured by deducting the first reading from the second.

3.2.3.3 Germination Assay

Glass slides were overlaid with 20 µL 1% agarose in milliQ water and covered with a coverslip until dry and cool to produce a thin, even surface. The coverslips were removed and the slides inoculated with 20 µL of a 10^6 spores.ml^{-1} spore solution. Slides were placed in a humid chamber and incubated in the dark at 20 °C. At 24 h incubation the slides were examined under a light microscope and the ratio of

germinated to ungerminated spores was calculated. Spores were considered to be germinated if they had a visible germ tube.

3.2.4 Enzyme Assays

3.2.4.1 Preparation of Mycelium from Liquid Culture

Spores were harvested from each of the strains under investigation and 10^7 spores were inoculated into 100 mL minimal media in a 250 mL flask and incubated for 3 days in the dark at 20 °C with shaking at 140 rpm. Cultures were harvested and centrifuged for 10 min at 3000 g at 4 °C. Pellets were washed in 50 mM Tris-HCl pH 7.5 for 10 min at 3000 g at 4 °C. The supernatant was discarded and mycelium was snap frozen by placing the tubes in liquid nitrogen. Samples were freeze dried overnight in Savant FDC206 freeze-drying chamber (Savant Scientific Instruments, Farmingdale, NY) attached to an Heto Maxi-Dry Lyo freeze dryer (Heto-Holten, Allerød, Denmark) and tubes were replaced in liquid nitrogen and stored at -80 °C. Each sample was ground in a sterile mortar and pestle containing 5-10 mL liquid nitrogen. Once the liquid nitrogen had evaporated the tissue was further ground and resuspended in 2 mL 50 mM Tris-HCl pH 7.5. The sample was transferred to a 2 mL eppendorf tube, centrifuged at 20800 g for 45 min at 4 °C, and kept on ice thereafter. The proteins were desalted using PD-10 Desalting Columns (GE Healthcare, Uppsala, Sweden) as per the manufacturer's instructions. Briefly, the PD10 columns were equilibrated by eluting 25 mL 50 mM Tris-HCl pH 7.5 buffer and discarding the flow-through. The centrifuged supernatant was made up to 2.5 mL with 50 mM Tris-HCl pH 7.5 buffer and applied to the equilibrated column with the flow-through being

discarded. A further 3.5 mL buffer was applied to the column and the eluent collected and kept on ice for enzyme assay.

3.2.4.2 Determination of Protein Concentration

The protein concentration in the samples was determined using a modified bicinchoninic acid (BCA) method (Smith *et al.*, 1985). Briefly, a fresh 50:1 BCA:$CuSO_4$ working solution was made up. Each sample was assayed in triplicate with 1 mL working solution and up to 50 µL desalted cell free extract. Where dilution was required the sample was made up to 50 µL with milliQ water. A blank comprising 50 µL sterile water was included. Bovine serum albumin (BSA) dilutions of 0, 0.2, 0.4, 0.6, 0.8 and 1.0 mg BSA.mL^{-1} milliQ water were prepared in triplicate to formulate a protein standard curve. The samples were incubated at 37 °C for 30 min and absorbance at A_{562} was measured on a Lambda 25 UV/VIS Spectrophotometer (Perkin Elmer) running Perkin Elmer™ UV Winlab Version 2.85.04. Absorbance readings for blanks were subtracted from those of tests to produce a net absorbance and the BSA protein standard curve used to determine protein concentration in mg protein.mL^{-1} CFE.

3.2.4.3 Measurement of Relative Enzyme Activity

Samples were maintained on ice between all the following procedures. Cuvettes for spectrophotometric assay were prepared in triplicate for each sample. Each cuvette contained 50 µL desalted sample supernatant, unless otherwise stated, and the reagents and volumes of each used for each enzyme assayed are as detailed

below. Each assay volume was made up to 1 mL with milliQ water. Enzyme substrates were not added until the samples were equilibrated and in the case of negative controls, water was added instead of the substrate. The oxidation of NADH/NADPH or reduction of NAD^+/$NADP^+$ was measured at 340 nm to determine activity in terms of $U.mL^{-1}$ extract. One unit of activity was defined as the amount of enzyme required to oxidise 1 μmol NADH/NADPH in 1 min, or to reduce 1 μmol NAD^+/$NADP^+$ in 1 min at 30 ºC.

3.2.4.3.1 $NADP^+$-dependent glucose 6-phosphate oxidation (glucose 6-phosphate dehydrogenase)

This protocol was based on that of Langdon (1966). Each reaction volume consisted of 500 μL 0.1 M Tris-HCl buffer pH 7.5, 45 μL 20 mM $NADP^+$, 140 μL 0.1 M $MgCl_2$, 50 μL CFE supernatant (milliQ water for control) and made up to 960 μL with milliQ water. After equilibration 40 μL 50 mM glucose 6-phosphate was added to start the reaction. Glucose 6-phosphate oxidation was measured by proxy using the reduction of $NADP^+$ as determined by change in absorbance at 340 nm.

3.2.4.3.2 NADPH-dependent fructose reduction (mannitol dehydrogenase)

This protocol was based on Noeldner *et al*. (1994). Each reaction volume consisted of 500 μL 0.1 M Tris-HCl buffer pH 7.5, 10 μL 25 mM NADPH, 50 μL CFE supernatant (milliQ water for control) and made up to 667 μL with milliQ water. After equilibration 333 μL 2.4 M fructose was added to start the reaction. Fructose

reduction was measured by proxy using the oxidation of NADPH as determined by change in absorbance at 340 nm.

3.2.4.3.3 $NADP^+$-dependent mannitol oxidation (mannitol dehydrogenase)

This protocol was based on Trail and Xu (2002). Each reaction volume consisted of 200 µL 0.5 M Tris-HCl buffer pH 9.0, 100 µL 20 mM $NADP^+$, 50 µL CFE supernatant (milliQ water for control) and made up to 500 µL with milliQ water. After equilibration 500 µL 0.8 M mannitol was added to start the reaction. Mannitol oxidation was measured by proxy using the reduction of $NADP^+$ as determined by change in absorbance at 340 nm.

3.2.4.3.4 NADH-dependent fructose reduction (NAD-mannitol dehydrogenase)

This protocol was based on the NADPH-dependent fructose reduction assay above. Each reaction volume consisted of 500 µL 0.1 M Tris-HCl buffer pH 5.95, 10 µL 25 mM NADH, 50 µL CFE supernatant (milliQ water for control) and made up to 667 µL with milliQ water. After equilibration 333 µL 2.4 M fructose was added to start the reaction. Fructose reduction was measured by proxy using the oxidation of NADH as determined by change in absorbance at 340 nm.

3.2.4.3.5 NAD^+-dependent mannitol oxidation (NAD-mannitol dehydrogenase)

This protocol was based on the $NADP^+$-dependent mannitol oxidation assay above. Each reaction volume consisted of 250 µL 0.2 M Tris-HCl buffer pH 9.0, 36

μL 10 mM NAD^+, 50 μL CFE supernatant (milliQ water for control) and made up to 875 μL with milliQ water. After equilibration 125 μL 0.8 M mannitol was added to start the reaction. Mannitol oxidation was measured by proxy using the reduction of NAD^+ as determined by change in absorbance at 340 nm.

3.2.4.3.6 NAD^+-dependent sorbitol oxidation (sorbitol dehydrogenase)

This protocol was based on the $NADP^+$-dependent mannitol oxidation assay above. Each reaction volume consisted of 250 μL 0.2 M Tris-HCl buffer pH 9.0, 36 μL 10 mM NAD^+, 50 μL CFE supernatant (milliQ water for control) and made up to 875 μL with milliQ water. After equilibration 125 μL 0.8 M sorbitol was added to start the reaction. Sorbitol oxidation was measured by proxy using the reduction of NAD^+ as determined by change in absorbance at 340 nm.

3.2.4.3.7 NADH-dependent fructose 6-phosphate reduction (mannitol 1-phosphate dehydrogenase)

This protocol was based on Solomon *et al.* (2005a). Each reaction volume consisted of 500 μL 0.1 M Tris-HCl buffer pH 7.5, 10 μL 25 mM NADH, 50 μL CFE supernatant (milliQ water for control) and made up to 900 μL with milliQ water. After equilibration 100 μL 20 mM fructose 6-phosphate was added to start the reaction. Fructose 6-phosphate reduction was measured by proxy using the oxidation of NADH as determined by change in absorbance at 340 nm.

3.2.4.3.8 NAD^+-dependent mannitol 1-phosphate oxidation (mannitol 1-phosphate dehydrogenase)

This protocol was based on Kiser and Niehaus (1981). Each reaction volume consisted of 500 µL 0.1 M Tris-HCl buffer pH 7.5, 25 µL 10 mM NAD^+, 50 µL CFE supernatant (milliQ water for control) and made up to 900 µL with milliQ water. After equilibration 100 µL 10 mM mannitol 1-phosphate was added to start the reaction. Mannitol 1-phosphate oxidation was measured by proxy using the reduction of NAD^+ as determined by change in absorbance at 340 nm.

3.2.4.4 Calculation of Specific Enzyme Activity

Specific enzyme activity was calculated by dividing the relative enzyme activity (U.mL^{-1} extract) by the protein concentration (mg protein.mL^{-1} extract) to produce specific activity in U.mg $protein^{-1}$.

3.2.5 Stress Tolerance Assays

3.2.5.1 Osmotic Stress Assay

Selected strains were prepared as for the liquid growth assays above. Liquid media consisted of minimal medium with the carbon replaced with glucose at a final concentration of 25 mM and supplemented with 0, 0.25, 0.5, 0.75 and 1.0 M NaCl (Merck Pty Ltd, Kilsyth, Victoria).

3.2.5.2 Oxidative Stress Assay

Selected strains were prepared as for the liquid growth assays above. Liquid media consisted of minimal medium with the carbon replaced with glucose at a final concentration of 25 mM and supplemented with 0 mM, 0.1 nM, 0.33 nM, 0.10 μM, 0.33 μM, 0.10 mM and 0.33 mM tert-butyl hydroperoxide (Sigma-Aldrich, St. Louis, USA) as an oxidant.

3.2.6 Pathogenicity Assays

3.2.6.1 Detached Leaf Assay

Detached leaf assays were set up using a method modified from that described in Benedikz *et al*. (1981). Leaves were harvested from wheat cv. Amery plants approximately 2 weeks after potting. Harvesting was confined to the first true leaf. The first 2 cm from the tip were cut off and the next 4 cm of blade were used for the assay. Trimmed leaves were placed vein down onto a benzimidazole agar plate with the two ends being embedded into the agar using a blunt forceps and sterile cotton tip.

Leaves were prepared in triplicate for each treatment. Leaves were inoculated in the centre of the blade with a 5 μL droplet of 10^6 spores.mL^{-1} resuspended in 0.02 % Tween 20. Once inoculated, plates were wrapped with clingfilm and incubated at 23 °C under an alternating light regime of 12 h Lumilux® Plus Eco (L 36W/21-840, Osram, Germany) fluorescent lighting and 12 h dark. Leaves were monitored every 1-

2 days for chlorosis and appearance of lesions, and lesions were measured by a caliper. Pycnidia were counted every 1-2 days.

3.2.6.2 Whole Plant Spray

Wheat cv. Amery seeds were surface sterilised and sown 8 seeds per pot, 8 pots per treatment, and grown for 2 weeks as described above (Section 2.4). On the day of spraying, spores were harvested for each strain of interest and 13 mL of 10^6 spores.mL^{-1} in 0.02% v/v Tween 20 was made up for each strain (13 mL 0.02% v/v Tween 20 for the negative control). The spore suspensions were kept on ice and 1 mL of each was removed and stored at 4 °C for an assay of ability to grow on different carbon sources as described above (Section 3.2.3.2**)**. Pots were numbered and placed randomly 8 pots to a tray, with the numbers for each treatment being noted. Each set of 8 pots was sprayed evenly with the remaining 12 mL spore suspension in an AirClean 600 Workstation (AirClean Systems, Australia) running at 270 Pa. The spore suspension was applied with a Vivair 300 Mini Compressor (Vivaz) attached to an SMC air brush sprayer at 140 kPa in two equal lots and allowed to dry between applications. After the second application, the tray was filled with water to about 1 cm deep, covered with a lid and sealed with Parafilm. The sealed trays were left for 2 days and then uncovered. An Ultrasonic Humidifier KT-100A (Ultrasonic, Taiwan) was used to maintain a moist environment in the growth chamber for the following 5 days. All other growth conditions were as set out above (Section 2.4). On the 7^{th} day post-inoculation the plants were subjected to blind scoring with scores assigned as per Figure 3.4.

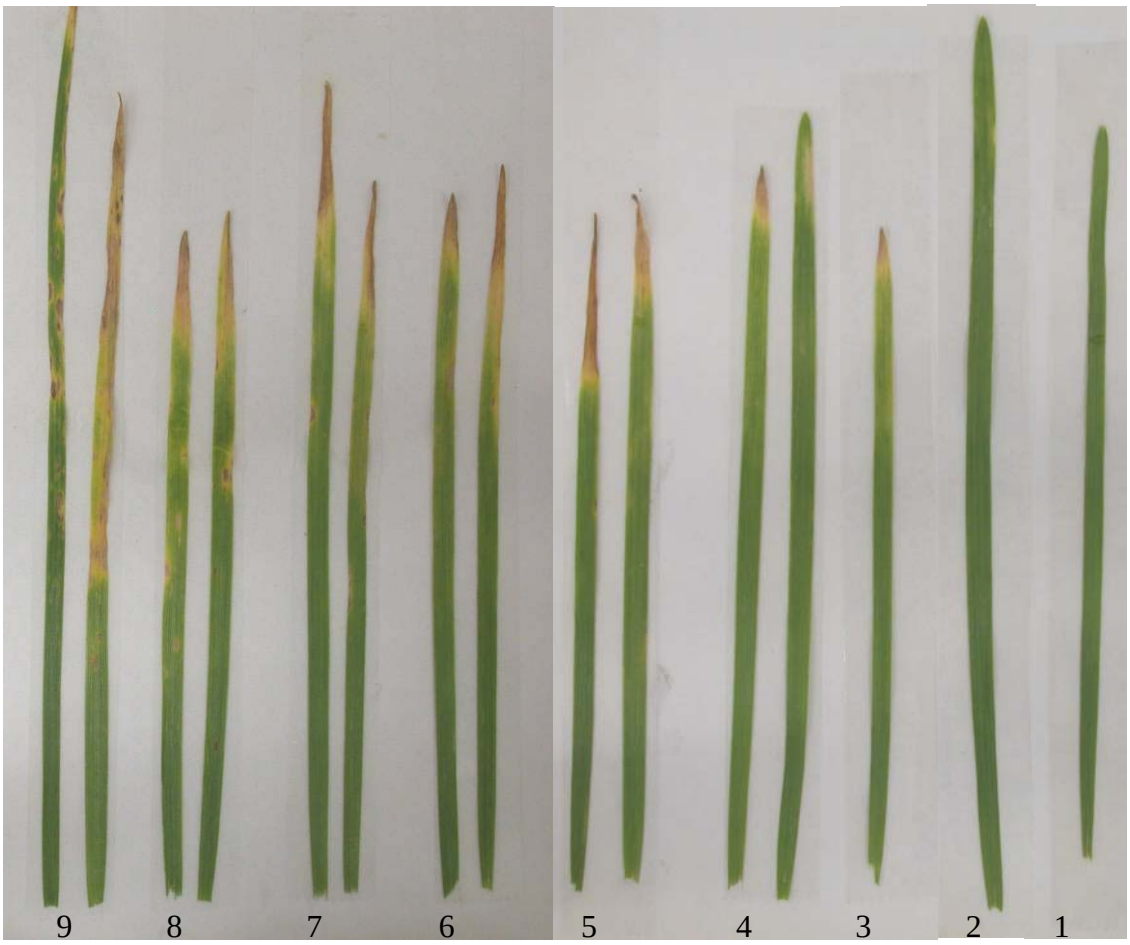

Figure 3.4: Score chart for assigning disease scores to wheat cv. Amery seedlings infected with strains of *Stagonospora nodorum.*

Source: ACNFP.

3.2.6.3 Latent Period Assay

On the day following the scoring for the whole plant spray, five leaves with lesions were harvested per treatment for a latent period assay. Only first true leaves were harvested, avoiding those which showed dead or yellowing tips, but which were representative of the treatment. Harvested leaves were trimmed and embedded in benzimidazole agar as described for detached leaf assay (Section 3.2.6.1 above). Leaves were examined daily and scored when the number of pycnidia at stage 4 or 5 (Figure 3.5) exceeded 50.

3.2.6.4 Microscopic Examination of Host Penetration

A detached leaf assay was set up as above (Section 3.2.6.1) using SN15 and the double mutant strain *mpd1mdh1-107*. Three leaves per strain were harvested at 1, 2, 4, 6 and 7 dpi. Leaves were cleared and stained with trypan blue using a method modified from those of Bruzzese and Hasan (1983) and Shipton and Brown (1962). Briefly, trypan staining solution was made up with 15 mL lactic acid, 15 g phenol, 28.8 mL 50% glycerol and 3.75 mL 0.4% trypan blue (all reagents from Sigma-Aldrich Inc., St. Louis, USA). Sampled leaves were boiled for 5 min in a 1:1 mixture of trypan blue staining solution and 100% ethanol, and left at room temperature overnight. Leaves were mounted and viewed under an Olympus BH-2 light microscope (Olympus Optical Co. Ltd.) and images captured with an Olympus DP12 camera (Olympus Optical Co. Ltd., Japan).

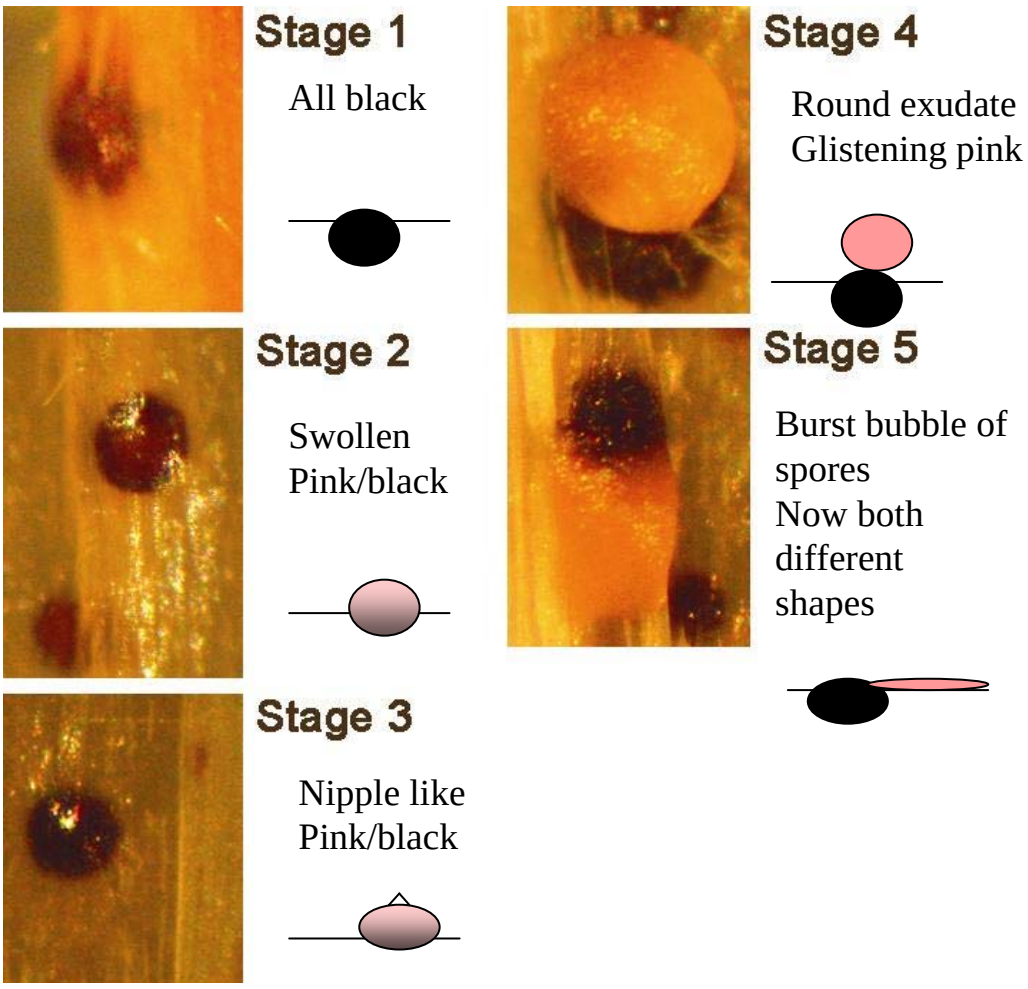

Figure 3.5: Criteria for assigning developmental stages in *Stagonospora nodorum* pycnidia on leaves of wheat cv. Amery.

Source: ACNFP.

3.2.7 Mannitol Supplementation Assays

3.2.7.1 In vitro *Response to Mannitol Supplementation*

3.2.7.1.1 In vitro *sporulation response to altered mannitol concentration*

The wild type strain SN15 and double mutant strain *mpd1mdh1-107* were selected for analysis. The background strain *mdh1-71* and ectopic double transformant *Mpd1mdh1-101* were included as controls. Pycnidiospores were harvested for each strain from CZV8CS agar cultures. Spore solutions of 10^6 spores.mL^{-1} were prepared and 20 µL inoculated per plate onto minimal medium agar plates supplemented with 0, 1, 3, 10, 30 and 100 mM mannitol. Each treatment was prepared in triplicate. Plates were incubated under the standard growth conditions and spores harvested and counted after 20 dpi.

The strains SN15, *mdh1-71*, *mpd1-1*, *mpd1mdh1-102*, *mpd1mdh1-107* and *Mpd1mdh1-101* were all grown on minimal media agar and minimal media agar supplemented with 3 mM mannitol. The inoculum for the *mpd1mdh1-107* strain was sourced from minimal medium plates which had been serially sub-cultured for 1, 2, and 3 generations to determine the effect of depleting residual mannitol from spores. All treatments were prepared in triplicate. Plates were incubated under the standard growth conditions and spores harvested and counted after 20 dpi.

3.2.7.1.2 Assay of mannitol content of spores

Pycnidiospores were harvested for SN15, *mdh1-71*, *mpd1-1* and *mpd1mpdh1-107* cultures grown on minimal medium plates, and on minimal medium plates supplemented with 3 mM mannitol. Spores were lyophilised overnight and metabolites extracted, derivatised, and analysed by GC-MS as per the protocol above for mannitol content.

3.2.7.2 In planta *Response to Mannitol Supplementation*

The wild type strain SN15 and the mutant strains *mdh1-71*, *mpd1-1*, *mpd1mdh1-102* and *mpd1mdh1-107* were selected for analysis. Three detached leaf assays were prepared as per the standard conditions with 4 replicate leaves per strain and Tween 20-inoculated and uninoculated controls. Once the inoculation droplets had all dried at 3 dpi, one of the DLAs had 5 µl drops of 3 mM mannitol added to each inoculation site on a daily basis. A second DLA had 5 µl drops of milliQ water added, and the third DLA had no addition. Lesion development was monitored and measured and the number of pycnidia per leaf counted every 1-2 days.

3.3 RESULTS

3.3.1 Isolation of the *mpd1mdh1* Double Mutant Strain

3.3.1.1 Transformation of Protoplasts

Two transformation experiments were conducted. The first experiment yielded a total of 7 colonies and the second yielded 47 colonies. In both experiments the negative control plates yielded zero colonies and the positive control plates exhibited confluent growth.

3.3.1.2 PCR Screening

PCR screening was performed on strains including the wild type (SN15), transformants harbouring either of the disruption constructs, and transformants harbouring both disruption constructs. The results of PCR using the mdhkoF/R primers and mpdkoF/R primers are shown (Figures 3.6 and 3.7 respectively). PCR screening identified 2 colonies out of the 54 transformants as putative homologous recombinants giving a recombination frequency of 3.7%. The putative homologous recombinants were designated *mpd1mdh1-102* and *mpd1mdh1-107*. A double transformant created during a previous experiment and designated *mpd1mdh1-51* (P. Solomon, pers. comm.) was included in the analysis. For the purposes of control and comparative analysis, an ectopic strain designated *Mpd1mdh1-101* was retained, as were the single mutant strains *mdh1-71* (used as the genetic background for the double mutant) and *mpd1-1*.

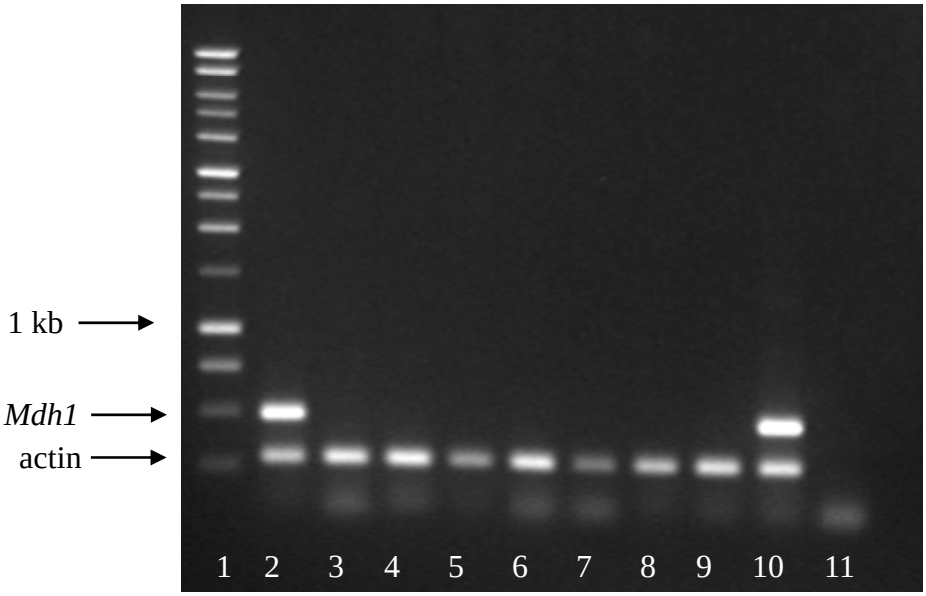

Figure 3.6: Duplex PCR amplification of gDNA from SN15 and mutant strains transformed with pGPSP-Mdh1 or having this construct as their background. PCR amplification was conducted using actinF/R primers (~300 bp) and mdhkoF/R primers (~461 bp), with an annealing temperature of 57 °C. Lanes: 1: 1 kb MW markers; 2: SN15; 3: *mdh1-71*; 4: *mdh1-73*; 5: *mdh1-79;* 6: *mpd1mdh1-51*; 7: *mpd1mdh1-102*; 8: *mpd1mdh1-107*; 9: *Mpd1mdh1-101*; 10: *Mdh1-63*; 11: negative

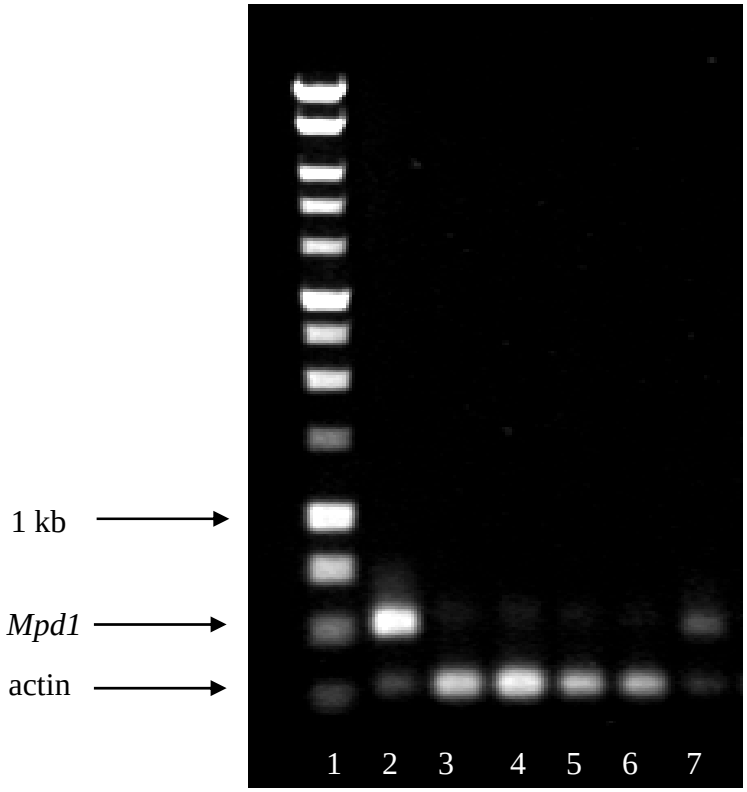

Figure 3.7: Duplex PCR amplification of gDNA from SN15 and mutant strains transformed with pGPSH-Mpd8. PCR amplification was conducted using actinF/R primers (~300 bp) and mpdkoF/R primers (~500 bp). Lanes: 1: 1 kb MW markers; 2: SN15; 3: *mpd1-1*; 4: *mpd1mdh1-51*; 5: *mpd1mdh1-102*; 6: *mpd1mdh1-107*; 7: *Mpd1mdh1-101*.

3.3.1.3 Southern Hybridisation

A PCR of SN15 gDNA using the MdhSOUTHF/R and MpdSOUTHF/R primers confirmed that the bands to be used as probes were of the predicted size (Figure 3.8). Southern analysis of selected strains using a probe homologous to the *Mpd1* gene demonstrated that this is a single-copy gene and that the doubly transformed strains *mpd1mdh1-102* and *mpd1mdh1-107* and the singly transformed control strain *mpd1-1* each contained a single insertion of the pGPSH-Mpd8 disruption construct (Figure 3.9A). The double transformant strain *mpd1mdh1-51* appeared to have multiple copies of the pGPSH-Mpd8 disruption construct and was discarded for further analysis. The hygromycin-/phleomycin-resistant ectopic mutant *Mpd1mdh1-101* showed the presence of the intact native gene.

Southern analysis of selected strains using a probe homologous to the *Mdh1* gene demonstrated that this is a single-copy gene and that the background strain *mdh1-71*, the singly transformed strains *mdh1-73* and *mdh1-79* and the doubly transformed strains *mpd1mdh1-102* and *mpd1mdh1-107* each had a single insertion of the pGPSP-Mdh1 disruption construct. The ectopic control strain *Mdh1-63* showed the presence of both the native and mutagenised versions of the gene (Figure 3.9B).

3.3.2 *In vitro* Phenotype

The strains SN15, *mdh1-71*, *mpd1-1*, *mpd1mdh1-107* and *Mpd1mdh1-101* were characterised on three different media as shown (Figure 3.10). The general morphology of colonies could be divided into three zones. Firstly there was an inner

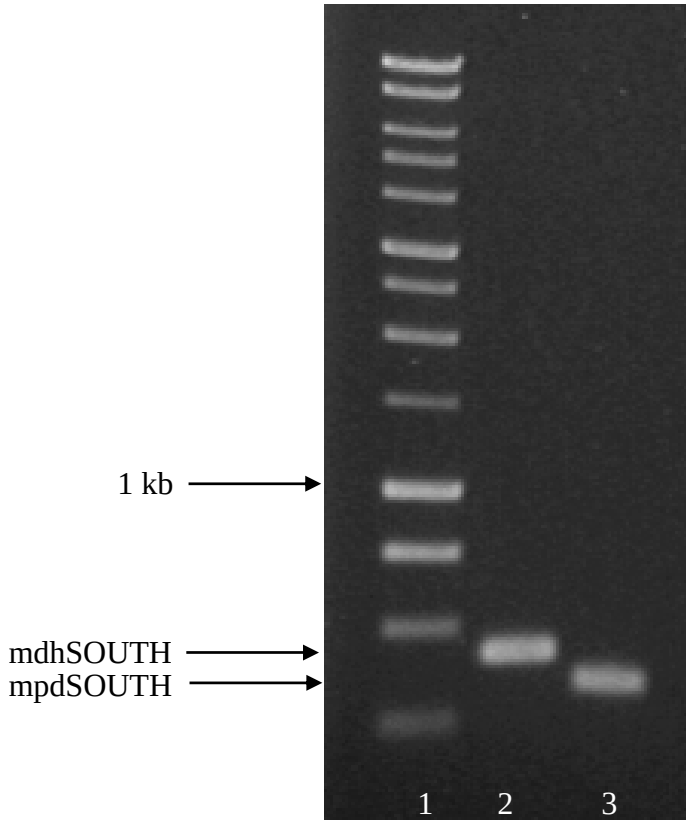

Figure 3.8: PCR amplification of gDNA from SN15 for use as a probe for Southern analysis. PCR amplification was conducted using mdhSOUTHF/R primers (~393 bp) or mpdSOUTHF/R (~311 bp). Lanes: 1: 1 kb MW markers; 2: mdhSOUTH band; 3: mpdSOUTH band.

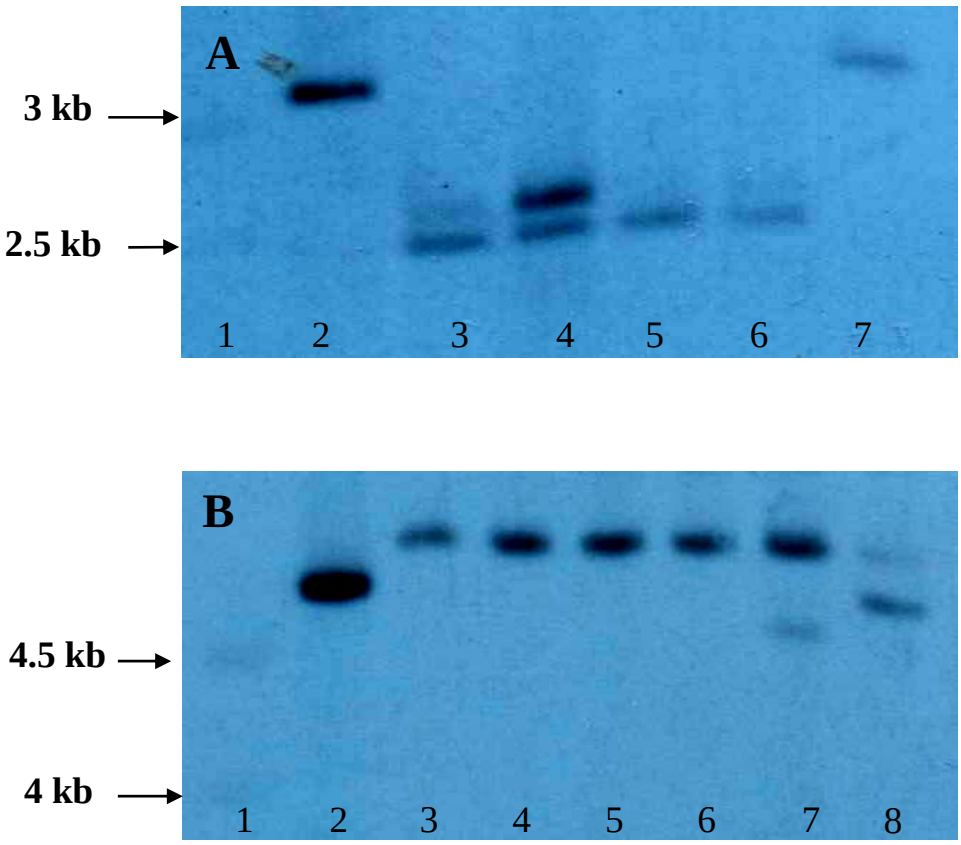

Figure 3.9:

A: Southern analysis of *Apa*I-digested gDNA transformed with the pGPSH-Mpd8 disruption construct, using probes homologous to *Mpd1*. Lane 1: MW markers; lane 2: SN15; lane 3: *mpd1-1*; lane 4: *mpd1mdh1-51*; lane 5: *mpd1mdh1-102*; lane 6: *mpd1mdh1-107*; lane 7: *Mpd1mdh1-101*.

B: Southern analysis of *Hind*III-digested gDNA transformed with the pGPSP-Mdh1 disruption construct, using probes homologous to *Mdh1*. Lane 1: MW markers; lane 2: SN15; lane 3: *mdh1-71*; lane 4: *mdh1-73*; lane 5: *mdh1-79*; lane 6: *mpd1mdh1-102*; lane 7: *mpd1mdh1-107*; lane 8: *Mdh1-63.*

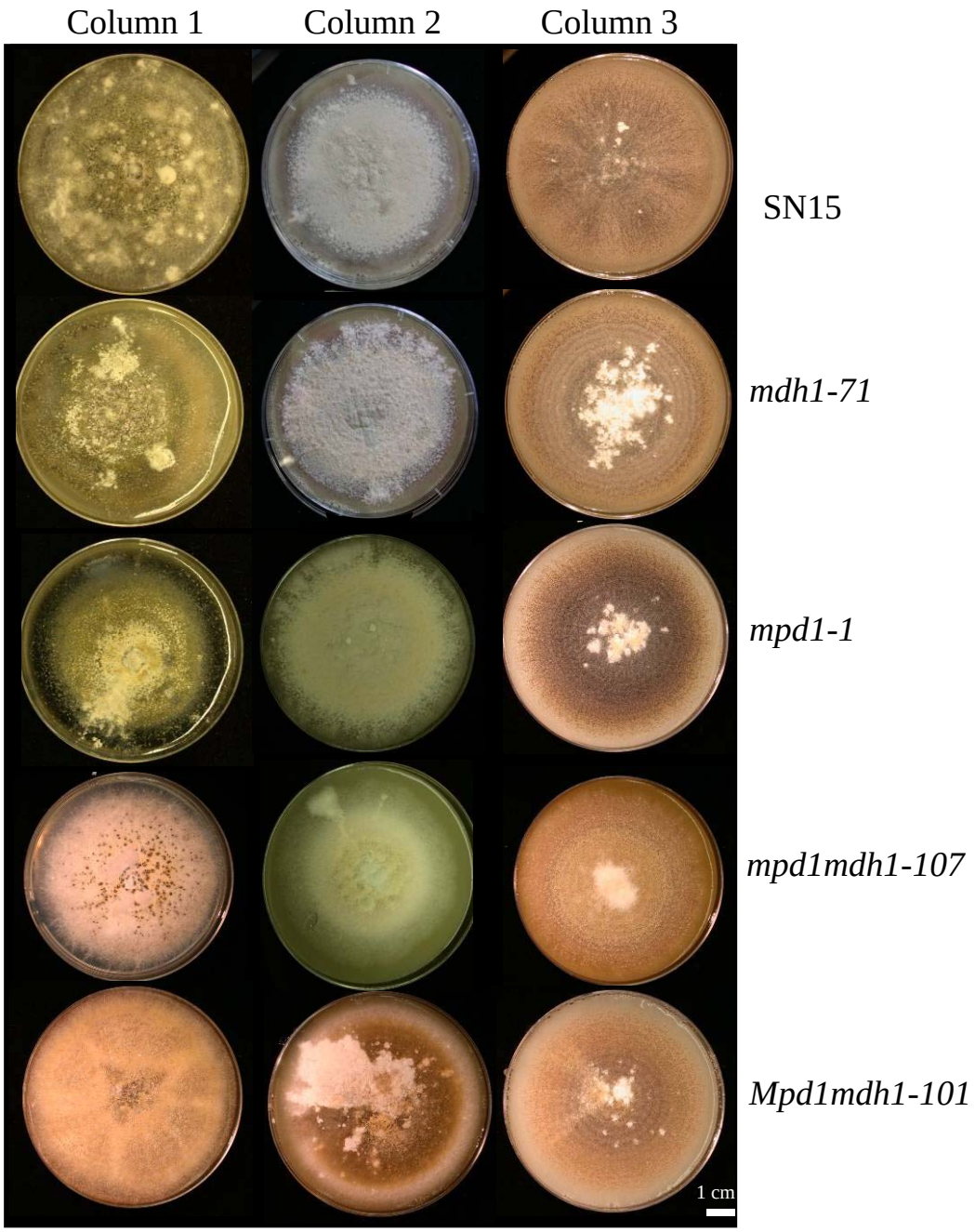

Figure 3.10: Phenotypic characterisation of strains of *Stagonospora nodorum* grown on three different media. Column 1: minimal medium (29 dpi); Column 2: V8-PDA (28 dpi); Column 3: CZV8CS (24 dpi).

zone consisting of the main body of the colony. This was often marked by dense pycnidiation with no diurnal rings discernable and could be covered with dense aerial mycelium. Next came an intermediate zone which tended to be covered in shorter aerial mycelium (~0.16 cm) if any was present, but with occasional denser clumps. This was the area in which diurnal rings of pycnidia occurred if they were present. These were formed in response to the light-dark cycle under which the fungus was grown. Finally, there was an outer zone or perimeter in which pycnidia were less dense or absent, and which usually had greatly reduced aerial mycelium. Specific differences between strains and media are as set out below.

3.3.2.1 Minimal Media Agar

When grown on minimal media agar plates SN15 produced colonies which were covered in short aerial mycelium which varied in density over the surface of the plate but formed no particular pattern. The colour of the aerial mycelium ranged from white, where it was thickest, to a creamy colour where it was thinner, and greyish where the thinner layer occurred over areas of dense pycnidiation. Pycnidia occurred ubiquitously but were denser in the main body of the colony which extended to within 6 mm of the edge of the petri dish. Thereafter, pycnidia occurred as randomly scattered single entities. Pycnidia located closer to the centre of the plate were oozing cirrhus.

The *mdh1-71* mutant was similar to the wild type although aerial mycelium seemed to be more evenly distributed and of a more even density. While some white clumps were evident, the general appearance was of a general tan/khaki colour

tending to more white/grey in the centre of the colony where pycnidiation was heaviest. The perimeter of the colony appeared to be less even and darker than was the case for SN15. Pycnidia towards the centre of the plate were observed to be oozing cirrhus.

The most outstanding feature of the *mpd1-1* mutant grown on minimal media was the darkness of the agar beneath the colony. The density of the aerial mycelium also decreased from the centre of the colony to the perimeter with a consequent change in the colour of the mycelium from white at the centre to tan/khaki over the intermediate portion and grey or translucent towards the perimeter. Pycnidia appeared to be abundant but were not oozing as profusely as SN15 at the centre of the colony. Moisture droplets were observed on the surface of the mycelium but it was not clear whether these were the result of condensation or produced by the mycelium.

The *mpd1mdh1-107* double mutant strain exhibited short white/beige aerial mycelium of uniform density apart from the occasional dense white clump. Pycnidia appeared abundant but rather than occurring ubiquitously in the main body of the colony as the other strains, they occurred in clumps, giving the colony a ‘spotted’ appearance. The pycnidia closer to the centre of the petri dish were more dense and were oozing cirrhus.

The ectopic strain *Mpd1mdh1-101* was similar to the background strain *mdh1-71*.

3.3.2.2 CZV8CS Agar

On CzV8CS agar plates, SN15 produced a circular, symmetrical colony which was basically flat on the plate. The perimeter of the colony consisted of a translucent region of dense, fine, short hyphae extending into the medium, followed by an opaque, non-sporulating zone of dense, pink-white mycelium. Behind this was a short, sparsely sporulating zone of orange-brown mycelium with sparse aerial hyphae, followed by the main body of the colony, consisting of dark brown diurnal rings of heavy sporulation beneath pink-white aerial hyphae which varied between being uniformly dense to being scattered and patchy.

The *mdh1-71* mutant was virtually identical to SN15 although seemed more prone to sectoring under these growth conditions, with areas of non-pycnidiation sometimes occurring within the intermediate zone. The *mpd1-1* mutant was also similar to SN15 but usually had a thicker covering of aerial mycelium over most of the colony.

The *mpd1mdh1-107* double mutant had quite a variable phenotype on this medium. This ranged from having dense pink-white mycelium around the central and intermediate portion of the colony with less dense hyphae around the perimeter, to having little aerial hyphae at all with the colony having bright pink colour, to having patchy pink aerial hyphae and with parts of the colony having no aerial hyphae and showing a ‘scalded skin’ look – bright red and wet looking. The strain also appeared to be prone to sectoring on this medium and some plates exhibited combinations of the above three phenotypes. The abundance and distribution of pycnidia was also very

variable with the first of the phenotypes above having normal looking pycnidia but being reduced in abundance, occurring around the main body of the colony only. The second phenotype produced more reduced numbers of pycnidia which tended to be scattered in no particular pattern. The ‘scalded skin’ phenotype produced almost no pycnidia.

The ectopic strain *Mpd1mdh1-101* was similar to the background strain *mdh1-71* except that while the amount of aerial mycelium was variable, it didn’t seem as prone to sectoring.

3.3.2.3 V8-PDA

Almost no aerial mycelium was observed for SN15 when grown on this medium. There was the occasional dense white clump, usually found towards the centre of the colony, but this was atypical for this strain. Pycnidia were dense and ubiquitous with formation of diurnal rings in the intermediate zone between the centre of the colony and the perimeter. Occasionally aerial mycelium occurred above the spaces between the pycnidial rings, but these were not profuse enough to render the pycnidial rings indistinguishable.

The *mdh1-71* mutant exhibited the same growth pattern as SN15 except that the pycnidial rings were obvious closer to the centre of the colony, perhaps suggesting that pycnidia were less dense than was the case for SN15. Formation of dense aerial hyphae towards the centre of the colony was more common but not universal. The *mpd1-1* mutant was also very similar to SN15 with commonly more aerial hyphae

formation towards the centre, and possibly slightly darker in colour – but not to the same extent as this strain growing on minimal media. Aerial hyphae in the outer part of the colony were sometime seen to occur as lines radiating out from the intermediate zone, but this was not universal.

The *mdh1mpd1-107* double mutant often had a dense pink-white clump of aerial mycelium at the very centre of the colony with short tan/khaki aerial hyphae generally occurring over the intermediate region. Where this central clump was absent, the centre consisted of clumps of pycnidia. In the outer 1/3 to ¼ of the colony aerial hyphae occurred as small, discrete pink-white spots roughly arranged in rings. Pycnidia were visible but did not appear to be as dense as the other three strains, although they may have been partly obscured by the aerial hyphae.

The ectopic strain *Mpd1mdh1-101* was similar to the background strain *mdh1-71*.

3.3.2.4 Mean Daily Growth Rates on Solid Medium

The strains SN15, *mdh1-71*, *mpd1-1* and *mpd1mdh1-107* were cultured on the three solid media above in triplicate. Colony diameters were measured on a daily basis from 3 to 14 days post inoculation when the majority of strains had grown to the limit of the plate. The daily growth rate was calculated for each strain on each medium and the Tukey-Kramer HSD test applied to compare the treatments (Figure 3.11). There was no statistically significant difference between the four strains on any one medium. There was also no statistically significant difference between the growth

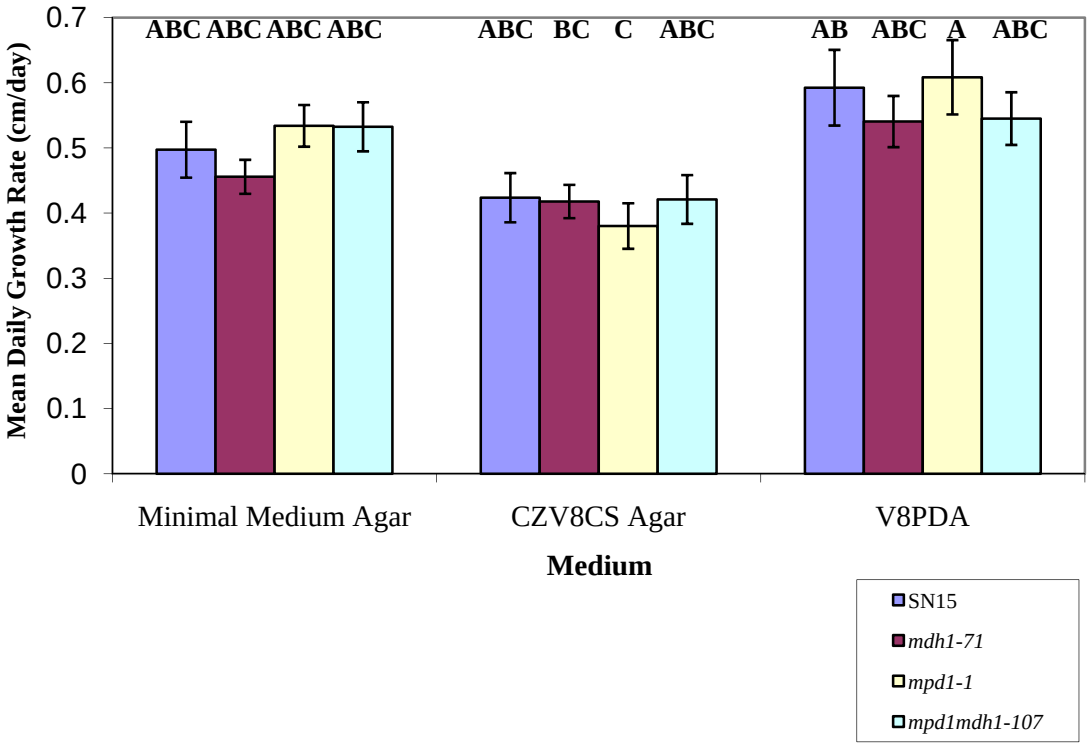

Figure 3.11: Mean daily growth rate (cm/day) (±SE) of strains of *Stagonospora nodorum* on solid media. Statistical significance was calculated using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. The means were calculated from the daily growth rates of triplicate samples measured daily from 3 dpi to 14 dpi. N=33.

rates on minimal medium agar or the other two solid media. SN15 and *mpd1-1* on V8-PDA had a significantly greater daily growth rate than *mpd1-1* on CZV8CS agar, and *mpd1-1* on V8-PDA also had a significantly greater daily growth rate than *mdh1-71* on CZV8CS agar.

3.3.2.5 Ability to Grow on Selected Carbon Sources

The strains SN15, *mdh1-71*, *mpd1-1* and *mpd1mdh1-107* were investigated. Growth on most media was equivalent for all strains with the exception that *mpd1mdh1-107* grew more poorly than the other strains on glucose and trehalose, while it showed equivalent growth on fructose and was intermediate on sucrose (Table 3.1). The three mutant strains grew poorly on mannitol compared to the wild type, with *mpd1mdh1-107* showing no growth on mannitol as a sole carbon source.

3.3.2.6 Germination Assay

The strains SN15, *mdh1-71*, *mpd1-1* and *mpd1mdh1-107* were investigated for the ability of pycnidiospores to germinate. The wild type strain and the double mutant were not significantly different from any other strains (Figure 3.12). The *mpd1-1* strain had a significantly higher mean percentage germination rate than the *mdh1-71* strain.

Table 3.1: Relative growth of *Stagonospora nodorum* strains SN15, *mdh1-71*, *mpd1-1* and *mpd1mdh1-107* on selected media in liquid culture. The base medium was minimal medium with no carbon source (MM-C) or no carbon or nitrogen source (MM-C-N) with supplements as indicated below.

Medium	**SN15**	***mdh1-71***	***mpd1-1***	***mpd1mdh1-107***
MM-C + 25 mM glucose	* * *	* * *	* * *	* *
MM-C + 25 mM fructose	* * *	* * *	* * *	* * *
MM-C + 25 mM sucrose	* * *	* * *	* * *	* *
MM-C + 25 mM trehalose	* * *	* * *	* * *	* *
MM-C + 25 mM mannitol	* * *	* *	*	-
MM-C-N + 1 $g.L^{-1}$ casamino acids	* * *	* * *	* * *	* * *
MM-C-N + 1 $g.L^{-1}$ casamino acids + 25 mM glucose	* * *	* * *	* * *	* * *
MM-C	-	-	-	-

* * * = good growth

* * = medium growth

* = poor growth

- = no growth

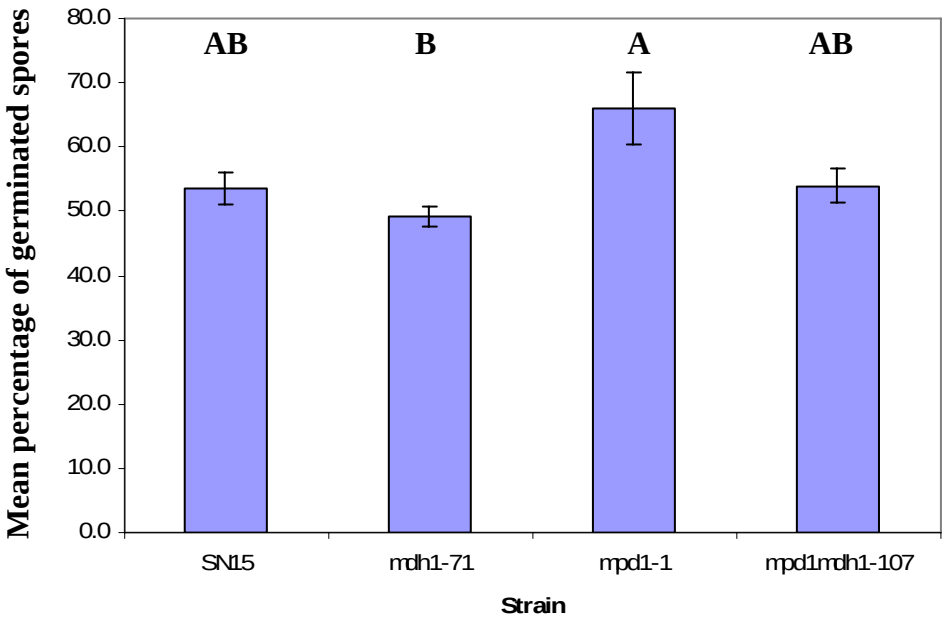

Figure 3.12: Mean percentage of germinated spores (±SE) for selected strains of *Stagonospora nodorum* at 24 hpi on 1% agarose. Statistical significance was calculated using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. N=3.

3.3.3 Enzyme Assays.

Enzyme assays were performed on desalted cell-free extracts of the strains SN15, *mdh1-71, mpd1-1, mpd1mdh1-107* and *Mpd1mdh1-101* (Table 3.2). Mannitol dehydrogenase activity was present in all strains in both directions except those strains in which the *Mdh1* gene was inactivated. Mannitol 1-phosphate exhibited the same behaviour except that fructose 6-phosphate reduction was not seen in the ectopic strain (although mannitol 1-phosphate oxidation was seen in this strain). NADH-dependent fructose reduction was observed in all strains and was an order of magnitude less than the NADPH-dependent fructose reduction where both activities were observed. There was, however, no NAD^+-dependent mannitol oxidation detected in any strain, while a low level of NAD^+-dependent sorbitol oxidation was observed in three of the strains. Glucose 6-phosphate dehydrogenase activity, used as a control to demonstrate enzyme activity in the samples, was detected in all strains.

3.3.4 Stress Tolerance Assays

3.3.4.1 Osmotic Stress Assay

There was no significant difference observed in the response of the strains to increasing concentrations of NaCl (Figure 3.13A).

Table 3.2: Specific enzyme activities for selected *Stagonospora nodorum* strains. All activities are shown as U/mg protein unless otherwise indicated.

Enzyme Activity	SN15	*mdh1-71*	*mpd1-1*	*mpd1mdh1-107*	*Mpd1mdh1-101*
NADPH-dependent fructose reduction	1.44 ± 0.39	nd	1.24 ± 0.42	nd	nd
$NADP^+$-dependent mannitol oxidation	11.8 ± 1.76	nd	3.07 ± 0.22	nd	nd
NADH-dependent fructose 6-phosphate reduction	0.21 ± 0.09	0.21 ± 0.09	nd	nd	nd†
NAD^+-dependent mannitol 1-phosphate oxidation*	5.36 ± 1.06	6.59 ± 2.11	nd	nd	1.31 ± 2.18
NADH-dependent fructose reduction	0.11 ± 0.01	0.10 ± 0.01	0.17 ± 0.02	0.15 ± 0.01	0.81 ± 0.05
NAD^+-dependent mannitol oxidation	nd	nd	nd	nd	nd
NAD^+-dependent sorbitol oxidation	0.02 ± 0.0	0.01 ± 0.01	nd	0.08 ± 0.04	nd
$NADP^+$-dependent glucose 6-phosphate oxidation	2.18 ± 0.21	0.64 ± 0.05	0.48 ± 0.06	1.27 ± 0.12	1.23 ± 0.20

nd = not detected

* Activity expressed in mU/mg protein

† While activity was not detected in this particular set of assays, activity equivalent to WT levels was seen in previous assays

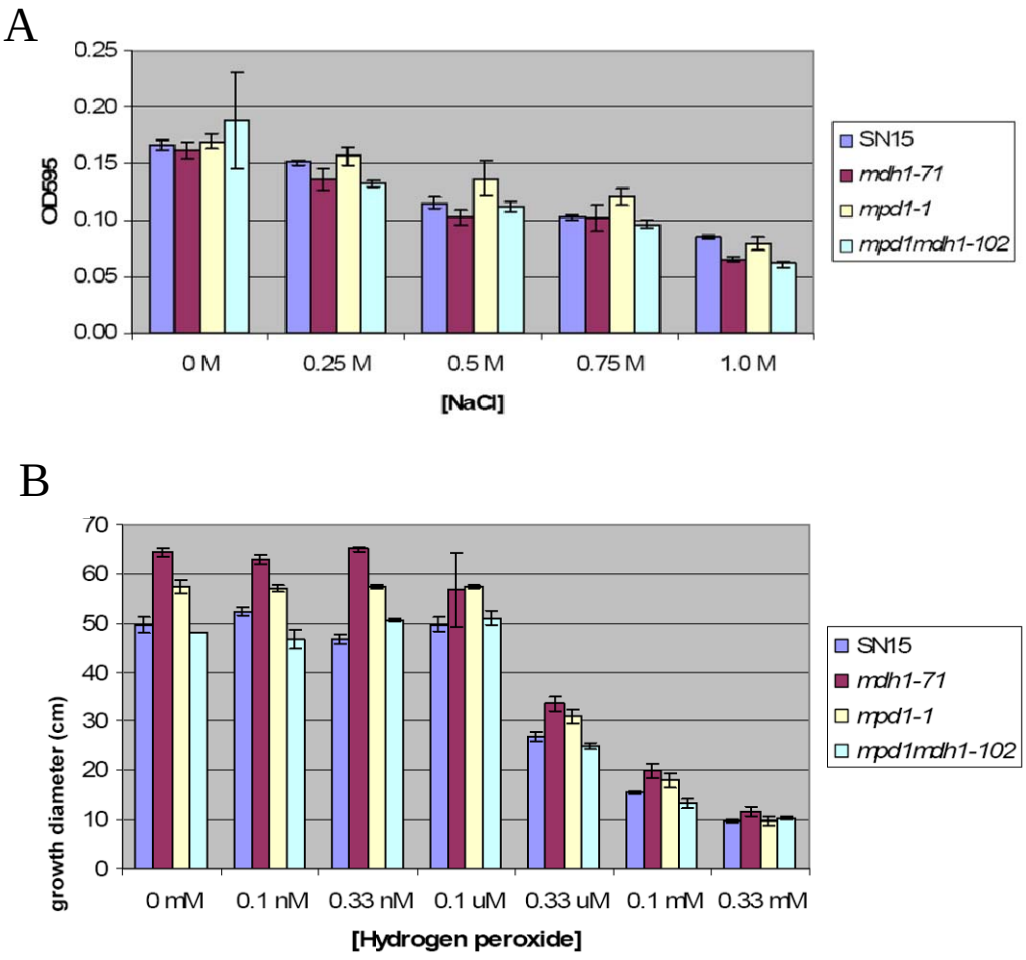

Figure 3.13: Assays of the ability of strains of *Stagonospora nodorum* to grow under conditions of osmotic stress (A) and oxidative stress (B). N=8.

3.3.4.2 Oxidative Stress Assay

There was no significant difference observed in the response of the strains to increasing concentrations of tert-butyl hydro-peroxide (Figure 3.13B).

3.3.5 Pathogenicity Assays

3.3.5.1 Detached Leaf Assay

A detached leaf assay was set up using the strains SN15, *mdh1-71*, *mpd1-1*, *mpd1mdh1-102*, *mpd1mdh1-107* and *Mpd1mdh1-101* with Tween 20-inoculated and uninoculated controls (Figures 3.14 and 3.15). Using the Tukey-Kramer Test to compare mean lesion formation for each day of measurement, the strains fell into four groups for the duration of the experiment, with only three exceptions. The strains SN15, *mdh1-71* and *Mpd1mdh1-101* exhibited the fastest rate of lesion formation, and their lesions were indistinguishable in appearance. The *mpd1-1* strain was intermediate in its rate of lesion formation between the wild type and the double mutants, and was significantly different to each. While it resembled a slower-progressing version of the wild type lesion, it produced no pycnidia (Figure 3.16). The double mutant strains both showed significantly reduced lesion formation, were darker in appearance, and formed no pycnidia (Figure 3.16). There was no lesion formation on either of the negative controls. The exceptions to the above groupings occurred at 4 dpi, where the *mdh1-71* strain was not significantly different from the *mpd1-1* strain; 6 dpi, where the *mpd1mdh1-107* strain was not significantly different

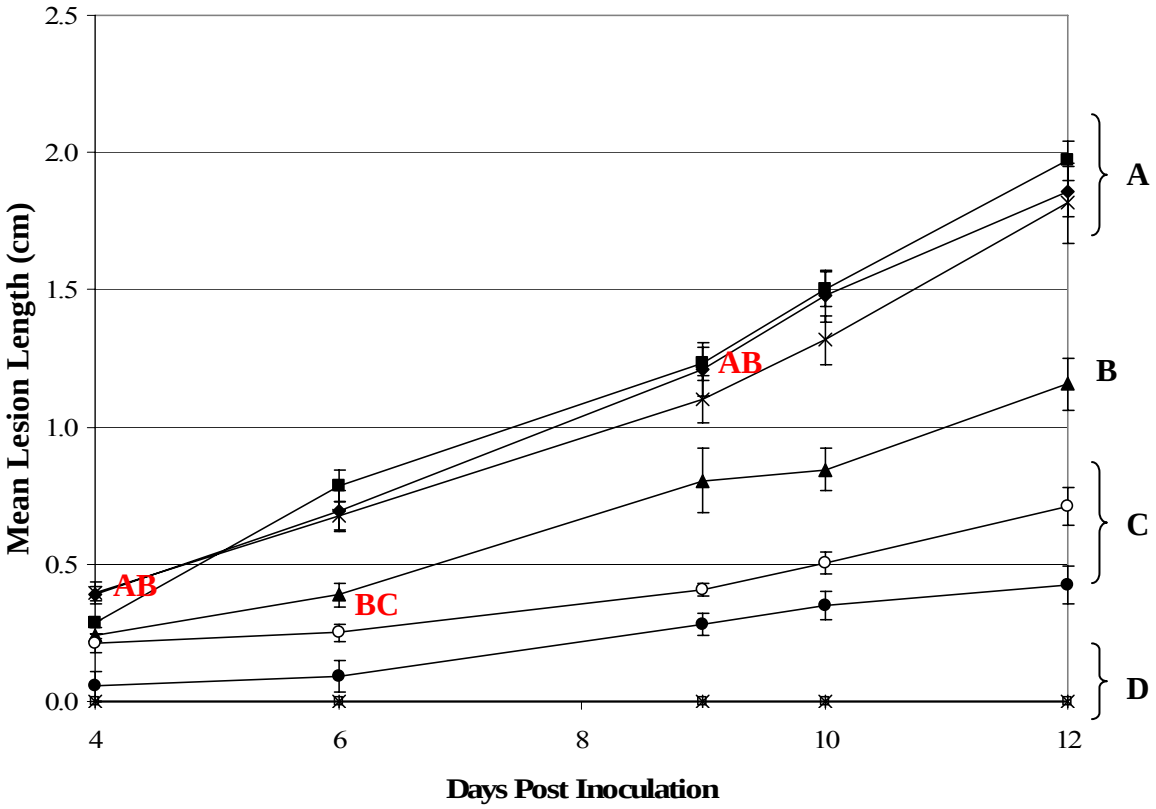

Figure 3.14: Mean lesion size (±SE) on detached wheat Amery leaves inoculated with SN15 (♦), *mdh1-71* (■), *mpd1-1* (▲), *mpd1mdh1-102* (●), *mpd1mdh1-107* (O) *Mpd1mdh1-101* (X), Tween control (*) and uninoculated control (□). Statistical significance was calculated for each day of measurement using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. The groups to which strains were assigned at 12 dpi are shown using black letters. Apart from three exceptions shown in red, all strains remained in the same groups for the duration of the experiment. N=4.

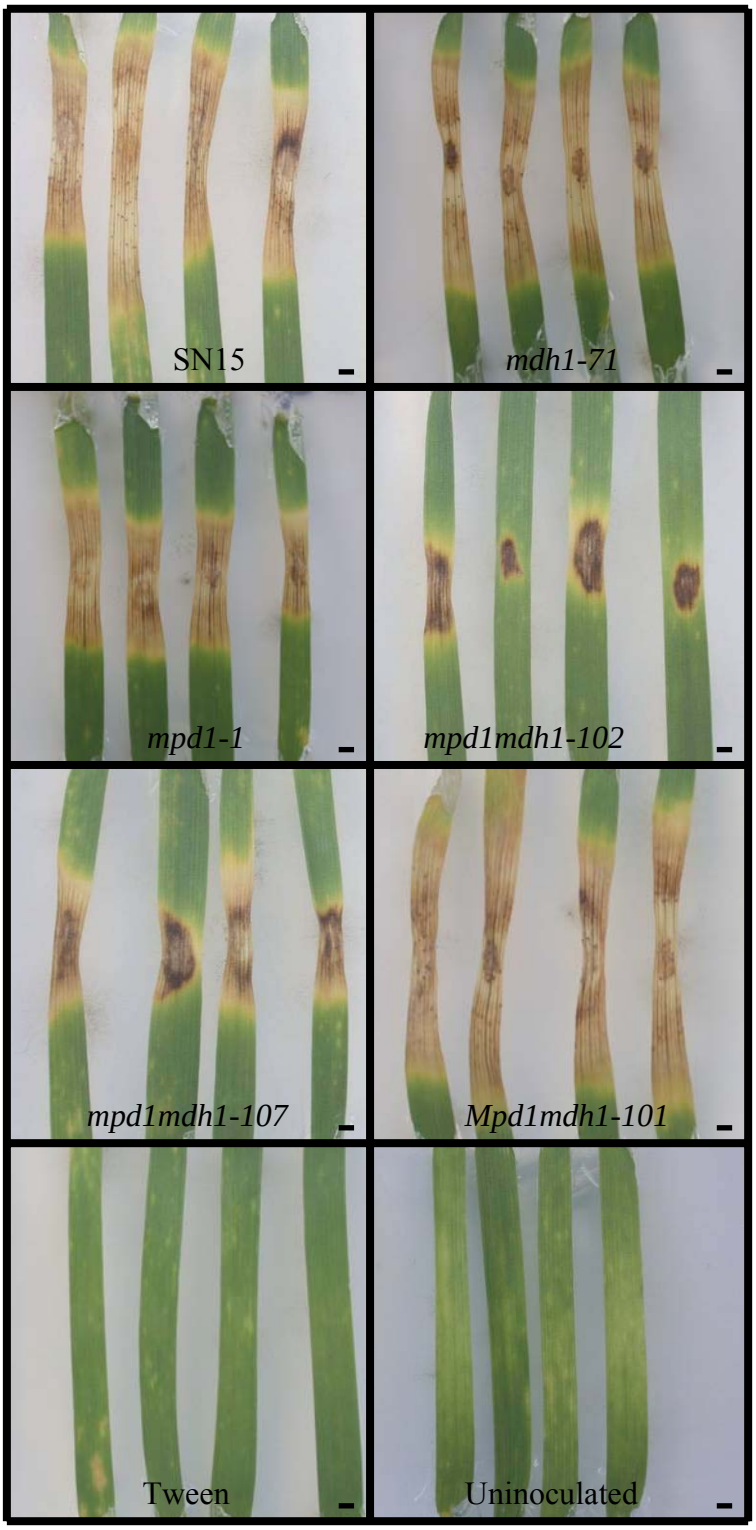

Figure 3.15: Detached leaf assay at 12 days post infection with strains of *Stagonospora nodorum* as noted above. (Bar = 1 mm).

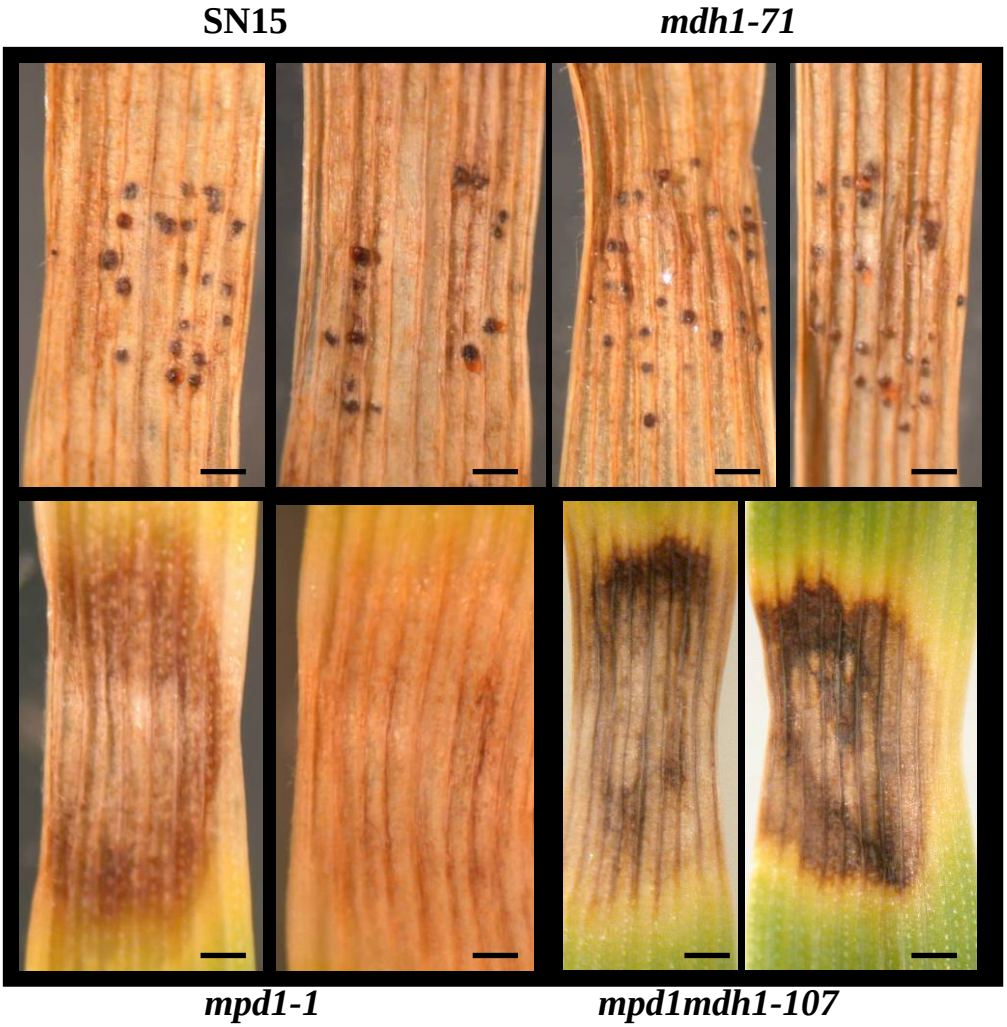

Figure 3.16: Lesion formation on a detached leaf assay at 12 days post-inoculation with selected strains of *Stagonospora nodorum* on wheat as noted above. Note the absence of pycnidia in the *mpd1* mutants. (Bar = 1 mm).

from the *mpd1-1* strain, and 9 dpi, where the *Mpd1mdh1-101* strain was not significantly different from the *mpd1-1* strain.

3.3.5.2 Whole Plant Spray

The results of a whole plant spray assay are presented (Figure 3.17). As determined by the Tukey-Kramer test, there was no significant difference in the ability of the mutants strains tested to cause disease from that of the wild type strain. The only significant difference noted between the strains was that the double mutant *mdh1mpd1-102* caused significantly less disease than its parent background strain *mdh1-71* and the ectopic double transformant strain *Mpd1mdh1-101*. The Tween 20-inoculated negative control exhibited a statistically lower mean disease score compared to all infected treatments.

3.3.5.3 Latent Period Assay

The strains SN15, *mdh1-71* and *Mpd1mdh1-101* all scored with more than 50 pycnidia at stage 4/5, five days after the LPA was set-up i.e. 12 days after the whole plant spray was inoculated. There were no pycnidia to be seen on the leaves inoculated with the strains *mpd1-1* or *mpd1mdh1-107*. There were 2 stage 4/5 pycnidia observed on one of the leaves inoculated with *mpd1mdh1-102*.

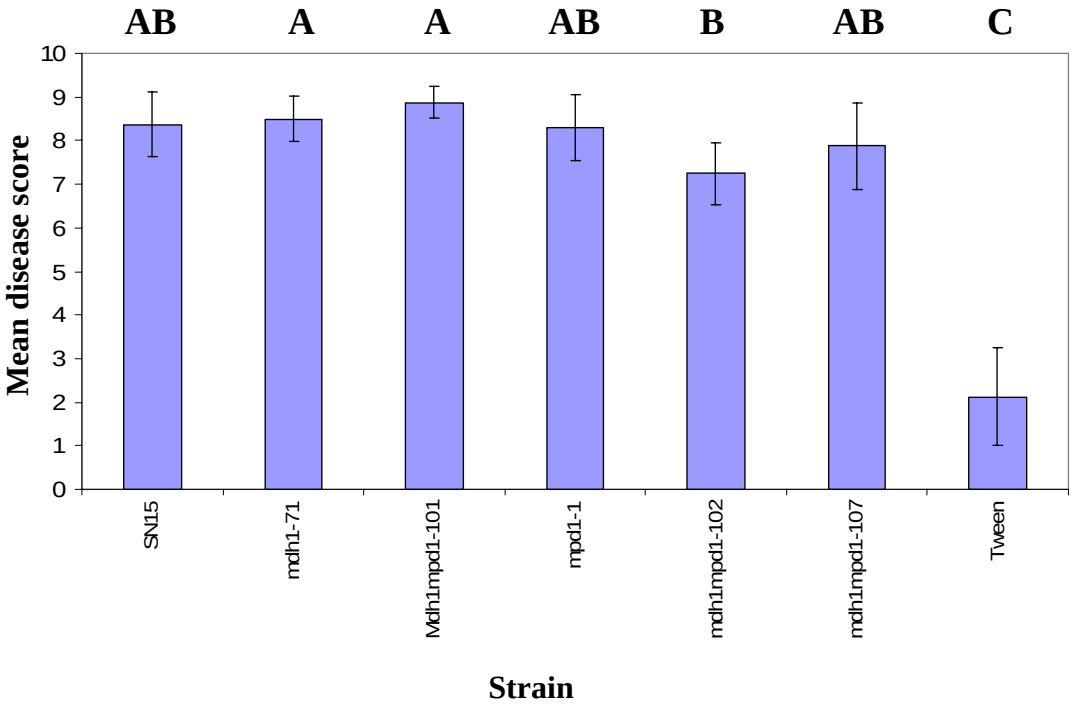

Figure 3.17: Mean disease scores (±SE) for wild type and selected mutant strains of *Stagonospora nodorum* from a whole plant spray pathogenicity assay. Statistical significance was calculated using the Tukey-Kramer HSD test, groups sharing a common letter are not significantly different. N=8 (N=7 for the strain *mpd1-1*).

3.3.5.4 Microscopic Examination of Host Penetration

The double mutant strain *mpd1mdh1-107* was compared with the wild type for its ability to invade the host via stomata or direct penetration of the cuticle by hyphae or hyphopodia. It was found to use all three methods of penetration (Figure 3.18).

3.3.6 Mannitol Supplementation Assays

3.3.6.1 In vitro *Response to Mannitol Supplementation*

3.3.6.1.1 In vitro *sporulation response to altered mannitol concentration*

The variability of the *mpd1mdh1-107* double mutant to produce pycnidia and spores was investigated by growing the strain on minimal media supplemented with 0, 1, 3, 10, 30 and 100 mM mannitol. Spore production was maximal at 3 mM mannitol (Figure 3.19A) and pycnidia formation was observed to increase with increasing concentration of mannitol up to 3 mM and to decrease thereafter (Figure 3.19B). The response of SN15, *mdh1-71* and *Mpd1mdh101* to changes in mannitol concentration were negligible by comparison.

When all strains under investigation were grown on minimal media agar with and without 3 mM mannitol supplementation, it was found that there was no effect upon strains with an intact *mpd1* gene, but that all *mpd1*-disrupted strains had spore counts increased to almost wild type levels by the addition of mannitol (Figure 3.20). The double mutant strain *mpd1mdh1-107* was subcultured for three generations onto

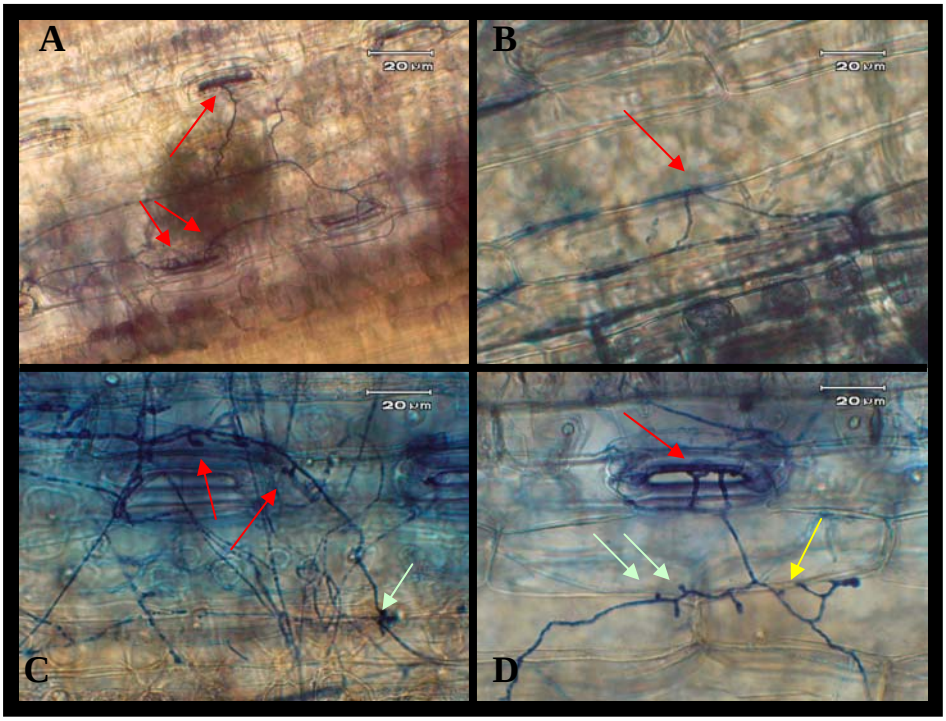

Figure 3.18: Trypan blue-stained lesions from detached leaves infected with *Stagonospora nodorum* strains SN15 and *mpd1mdh1-107*. Arrows indicate penetration attempts.

A: *mdp1mdh1-107* hyphae entering stomata (2 dpi)

B: *mdp1mdh1-107* hyphae penetrating host cuticle (2 dpi)

C: *mdp1mdh1-107* differentiating hyphopodia (green arrow shows a hyphopodium penetrating the host cuticle) (7 dpi)

D: SN15 (6 dpi) showing entry by stomate (red arrow), direct penetration by hyphae (yellow arrow), and formation of hyphopodia (green arrows)

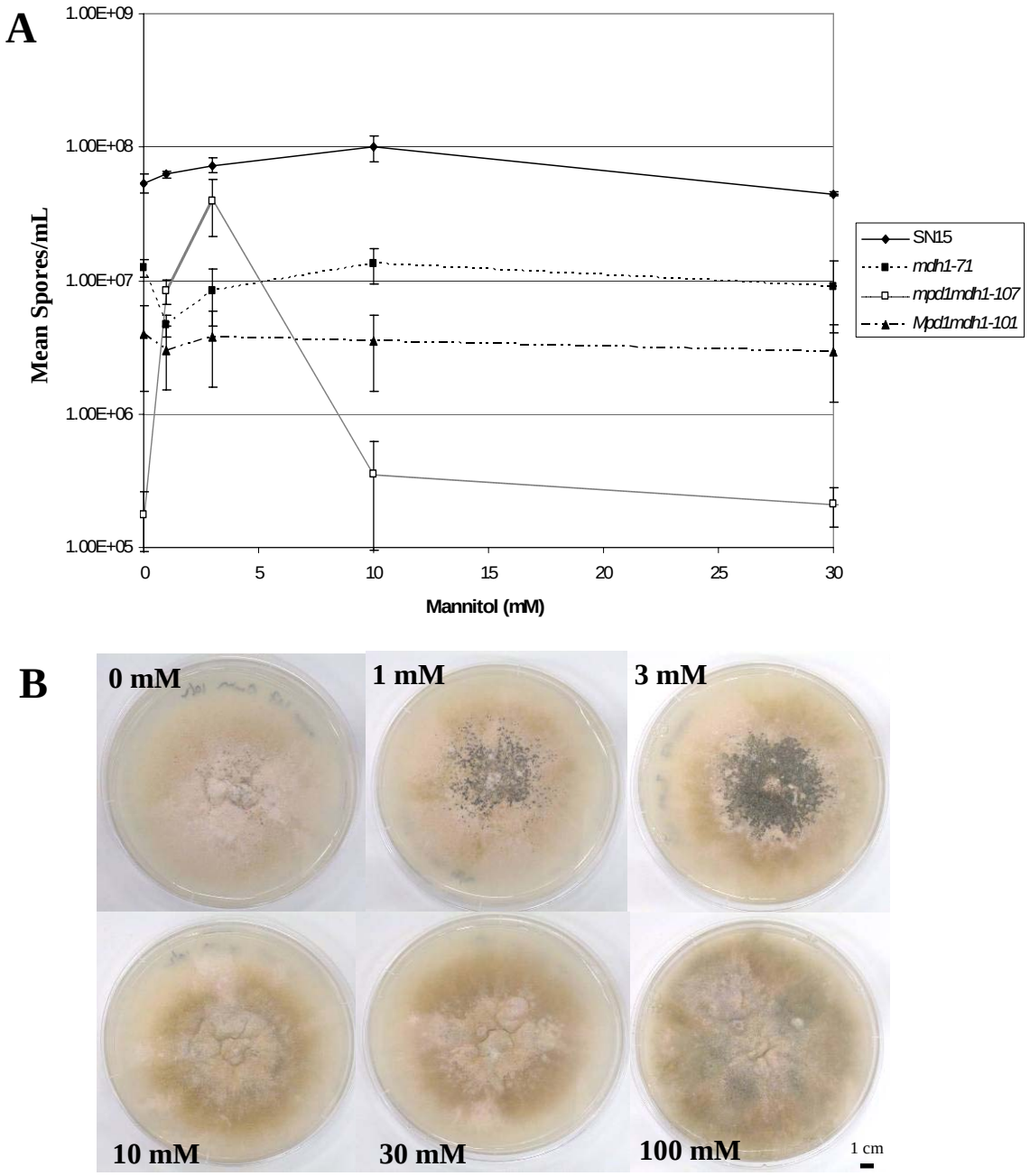

Figure 3.19:

A: The effect of mannitol supplementation upon sporulation of the strains SN15, *mdh1-71, mpd1mdh1-107* and *Mpd1mdh1-101*. Mean spores/mL (±SE) for strains grown on minimal media agar supplemented with 0, 1, 3, 10 and 30 mM mannitol are shown. N=3.

B: Pycnidia production by *mpd1mdh1-107* in response to changes in mannitol concentration in supplemented minimal media agar.

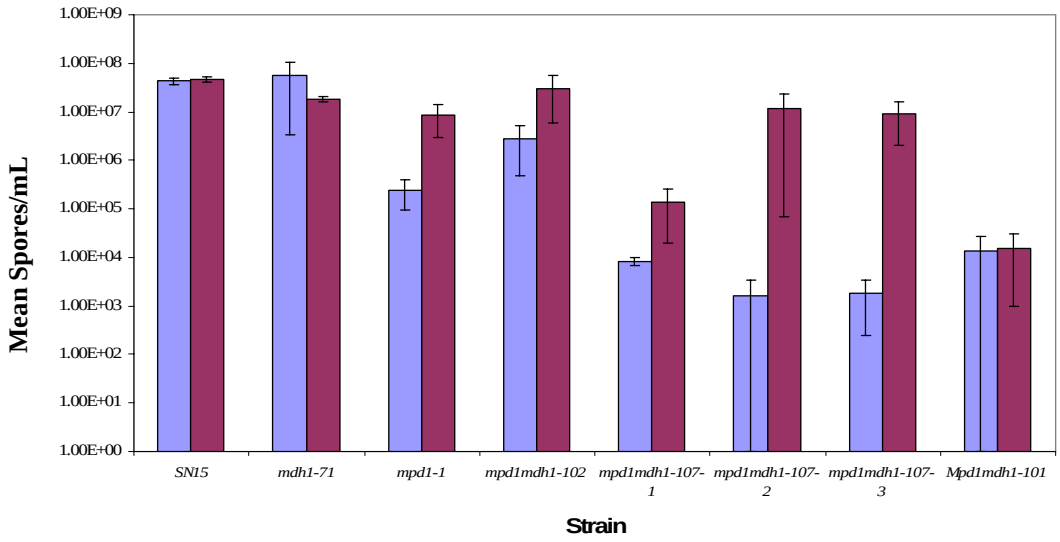

Figure 3.20: Comparison of mean spores/mL (±SE) for strains of *Stagonospora nodorum* as shown. Blue columns result from growth on minimal media agar. Purple columns result from growth on minimal media agar supplemented with 3 mM mannitol. The inoculum for the double mutant strain *mpd1mdh1-107* came from minimal medium agar plates on which the strain had been serially sub-cultured for 1, 2 and 3 generations as indicated by the suffix. N=3. Note that the y axis has a logarithmic scale.

the two media treatments prior to inoculation and while zero spores were recorded for some plates, sporulation was not completely abolished in all biological repeats for any of these generations.

3.3.6.1.2 Assay of mannitol content of spores

GC-MS analysis demonstrated that spores of SN15 cultured on minimal medium agar contained large amounts of mannitol, while the *mpd1*-disrupted mutants grown on the same medium had only traces to undetectable amounts of mannitol in their spores (Figure 3.21). When *mpd1mdh1-107* was grown on minimal media supplemented with 3 mM mannitol, large amounts of mannitol were detected. Due to the small numbers of spores obtained from the unsupplemented cultures of the *mpd1*-disrupted mutants, only qualitative comparisons could be made.

3.3.6.2 In Planta *Response to Mannitol Supplementation*

Addition of exogenous mannitol to lesions on leaves infected with SN15, *mdh1-71*, *mpd1-1* and *mpd1mdh1-107* in a detached leaf assay resulted in the formation of pycnidia on leaves infected by *mpd1mdh1-107* and *mpd1-1* by 12 dpi (Figure 3.22). While these pycnidia were not as abundant as those formed on leaves infected with SN15 or *mdh1-71*, and appeared to be smaller, it was the only condition in which pycnidia were produced in a DLA by the *mpd1*-disrupted strains. To exclude the possibility that there was cross-infection, pycnidia were picked off the leaves and inoculated onto media containing appropriate antibiotics. In all cases there was

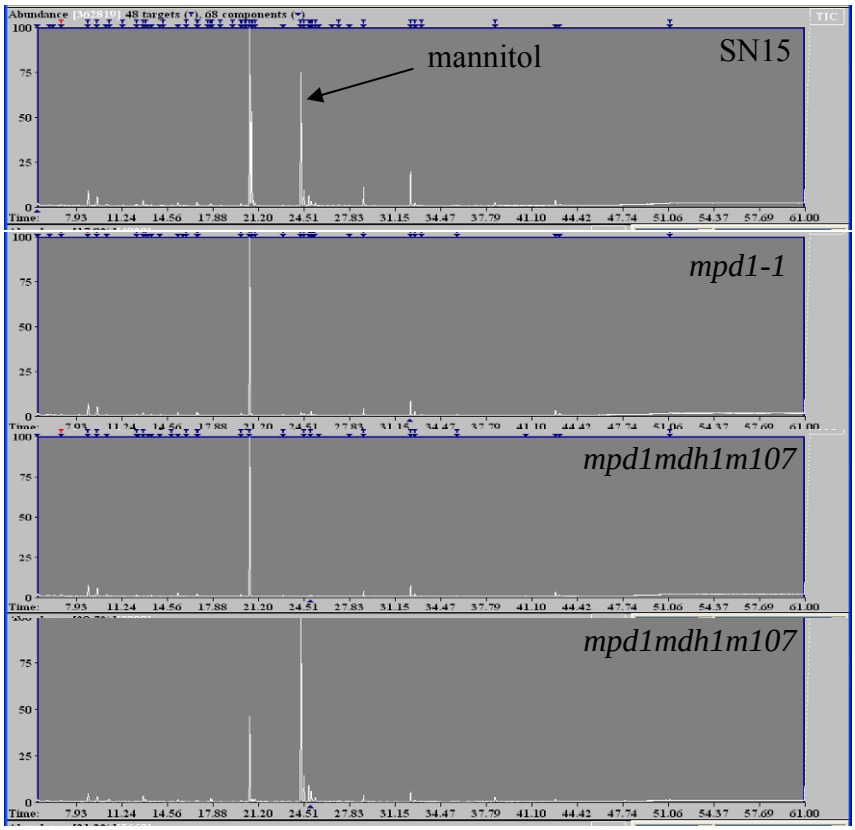

Figure 3.21: GC-MS chromatograms demonstrating the amount of mannitol present in spores of SN15, *mpd1-1* and *mpd1mdh1-107* harvested from minimal medium agar plates. The bottom chromatogram came from spores cultured on medium supplemented with 3 mM mannitol. The retention time of mannitol was 24.51 in these chromatograms.

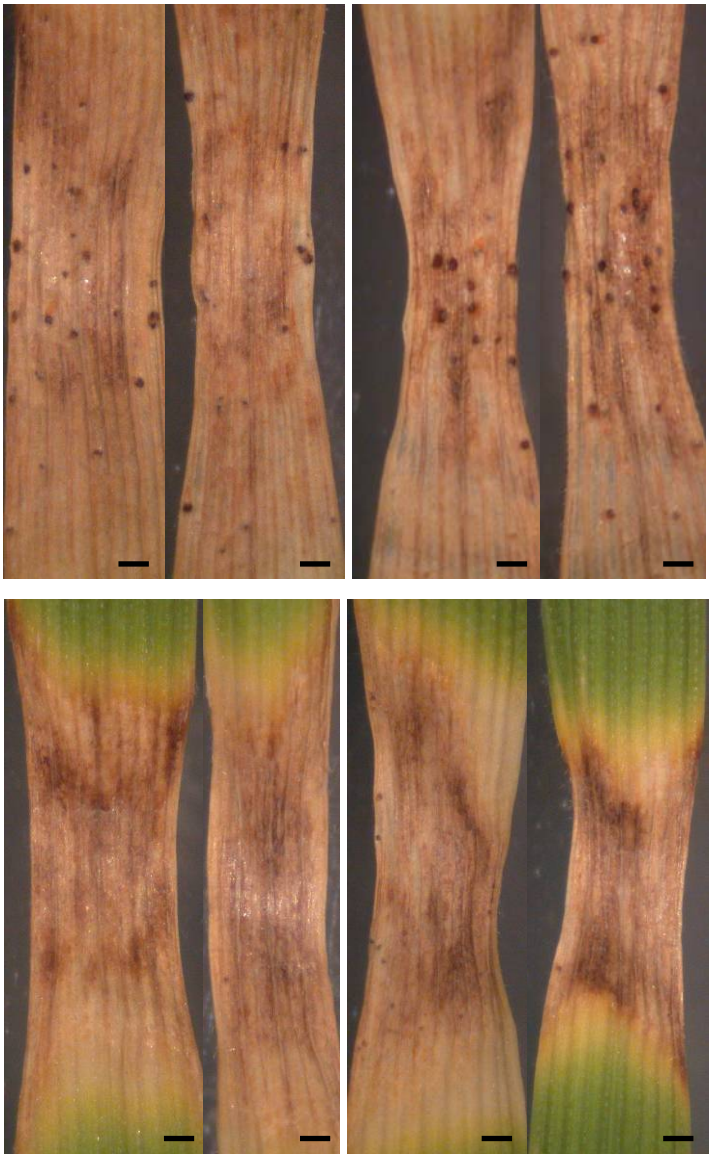

Figure 3.22: Chemical complementation of the *in planta* sporulation defect of the *Stagonospora nodorum* double mutant strain *mpd1mdh1-107*. Lesions were inoculated with 5 µL 3 mM mannitol on a daily basis from 3 days post infection (right). Mocks (left) were inoculated with 5 µL water. Top row: SN15; bottom row: *mpd1mdh1-107*. Pictures were taken at 12 dpi. (Bar = 1 mm).

growth on the plates, thus confirming that the pycnidia were formed by the strain used for the leaf infection.

3.4 DISCUSSION

3.4.1 Isolation of the *mpd1mdh1* Double Mutant Strain

The transformation rate of 3.7% observed in this study was comparable with the 3% homologous recombinant strains reported during the transformation of SN15 with the pGPSH-Mpd8 disruption construct (Solomon *et al*., 2006c). The recent release of the *S. nodorum* genome sequence (Hane *et al.*, 2007) has confirmed the Southern analysis result that the two genes of interest were single-copy. The gene encoding mannitol 2-dehydrogenase (*Mdh1*) is identified by the record SNOG_09898.2 and is found on scaffold 38. The gene encoding mannitol 1-phosphate dehydrogenase (*Mpd1*) is identified by the record SNOG_12666.2 and is found on scaffold 40.

3.4.2 Enzyme Assays

Cell-free extracts from all strains were positive for the constitutive enzyme glucose 6-phosphate dehydrogenase, demonstrating that there was active protein. Strains which had an intact *Mdh1* gene (SN15 and *mpd1-1*) demonstrated mannitol dehydrogenase activity in both the forward and reverse reaction. Strains which had an intact *Mpd1* gene (SN15, *mdh1-71* and *Mpd1mdh1-101*) demonstrated mannitol 1-phosphate dehydrogenase activity in both the forward and reverse directions, with the

exception of the ectopic strain. In this particular set of assays, the protein concentration of the CFE for the ectopic strain was less than half that of the other strains and the reduction of fructose 6-phosphate was not detected, although the oxidation of mannitol 1-phosphate was detected. Fructose 6-phosphate reduction was previously seen in this strain during the optimisation of the assay with an activity of 0.02 U/mg protein. Strains in which the *Mdh1* and/or *Mpd1* genes were inactivated demonstrated no activity for the disrupted gene product.

A putative NAD-dependent mannitol dehydrogenase activity was assayed. While activity was demonstrated in all strains using fructose as the substrate and NADH as the co-factor, there was no activity in any strain using mannitol as the substrate and NAD^+ as the co-factor. There was activity in some strains, however, using sorbitol as the substrate and NAD^+ as the co-factor. Sorbitol does not appear as a significant metabolite in *S. nodorum* in NMR or GC-MS analyses (Chapters 4 and 5, this study). It is possible that there is an alternative endogenous substrate for the oxidative reaction of this enzyme. Since this was not a focus of this study it was not investigated further.

3.4.3 Mannitol Synthesis can Occur by Two Pathways

The previous inactivation of the *Mpd1* gene in *S. nodorum* and *A. niger* showed that mutant strains were still able to synthesis mannitol, although to only 20-30% of wild type levels (Ruijter *et al.*, 2003; Solomon *et al.*, 2005a). The further characterisation of the *mdh1* mutant in *S. nodorum* showed that this strain is unaffected in mannitol synthesis (Chapter 5, this study). The double mutant strain was

unable to synthesise mannitol. Furthermore, no strain was able to oxidise mannitol using NAD^+ as a co-factor. These data demonstrate that there was no additional pathway of mannitol synthesis apart from the two under investigation. Secondly, mannitol synthesis can be facilitated by either of the "halves" of the mannitol cycle. This suggests that there is an as yet unidentified fructose 6-phosphate phosphatase gene in *S. nodorum*. The reduced ability of the *mpd1-1* mutant to accumulate mannitol suggests that synthesis of mannitol occurred primarily via mannitol 1-phosphate in *S. nodorum*. This is also consistent with the findings in *A. niger* (Ruijter *et al.*, 2003). It cannot be determined on the basis on these studies whether these pathways operate simultaneously *in vivo* or whether they may be subject to regulation.

3.4.4 Mannitol Catabolism is Facilitated Primarily via Mannitol 1-Phosphate

The *mpd1* inactivated strains were essentially unable to grow on mannitol as a sole carbon source indicating that the catabolic step of the mannitol cycle is unable to utilise mannitol in the absence of mannitol 1-phosphate dehydrogenase. It could be claimed that this was perhaps due to some catabolism-inhibiting compound produced when *Mdh1* is used as the sole pathway for mannitol synthesis. The *mdh1-71* strain, however, was able to grown on mannitol as a sole carbon source, albeit at a reduced rate compared to the wild type. This indicates that mannitol is catabolised primarily, perhaps exclusively via mannitol 1-phosphate. This further suggests that mannitol metabolism does not occur in an enzymatic cycle, as described above, in *S. nodorum*. The implication is that the step from mannitol 1-phosphate to mannitol, catalysed by mannitol 1-phosphate phosphatase must be reversible. The simplest reaction achieving this would be facilitated by a mannitol kinase. While activity for such an

enzyme has been described in bacteria (Klungsøyr, 1966; Mehta *et al.*, 1977), it has not been convincingly demonstrated in fungi (Lones and Peacock, 1964; Lee, 1967b; Strandberg, 1969; Adomako *et al.*, 1972).

Recent independent confirmation that these enzymes do not operate in a cycle in other fungi comes from studies in *A. niger* (Aguilar *et al.*, 2008). Gene expression studies with GFP and dTomato fusions to the promoters of *MpdA* and *MtdA* indicated that while *MpdA* is expressed in mycelium, *MtdA* expression is restricted to conidia.

3.4.5 Mannitol is Required for Asexual Sporulation

Previous studies have shown that *Mdh1* is dispensable for asexual sporulation, with mutant strains producing wild type levels of pycnidia and spores both *in vitro* and *in vivo* (Waters, 2004), while *Mpd1* was required for sporulation *in planta*, but on standard growth medium was able to sporulate normally *in vitro* (Solomon *et al.*, 2005a). Manipulation of the medium to exclude mannitol, and serial subculturing to deplete exogenously accumulated mannitol from spores revealed that *mpd1* strains were compromised in their ability to sporulate *in vitro*. This was partially compensated for in the *mpd1* strain, which was previously shown to be able to synthesise low levels of mannitol. However, sporulation in the non-mannitol synthesising double mutant strain was abolished. This deficiency could be chemically complemented, and addition of 3 mM mannitol to the growth medium restored sporulation to wild type levels. The double mutant was shown to be unable to sporulate *in planta*. Addition of exogenous mannitol to the leaf lesions was able to partially complement this deficiency, with pycnidia being produced, although

sporulation did not reach wild type levels. The germination assay demonstrated that spores produced by the various mutant strains were not significantly affected in germination rates from the wild type. The difference noted between the strains where *mpd1-1* spores recorded a significantly higher germination rate in 24 h than *mdh1-71* spores, did not explain the slower rate of lesion development of the former strain compared to the latter.

There is a clear requirement for mannitol in asexual sporulation, and this is the first conclusive evidence for a role for this compound in fungi. The mode of action cannot be determined from this study, however. Mannitol has previously been proposed as having a role in stress tolerance, since it has been shown to be a potent quencher of reactive oxygen species (Smirnoff and Cumbes, 1989). Fungal mutants in which mannitol production has been reduced have shown increased sensitivity to heat and/or osmotic stress (Chaturvedi *et al.*, 1996; Ruijter *et al.*, 2003). The *mpd1* mutants did not show a significantly different response to oxidative or osmotic stress, but there may be some other stress encountered during the infection process for which mannitol is required. It is also possible that the presence of mannitol may be involved in a sensory pathway involved in pycnidiogenesis, in a similar way to the proposed extracellular sporulation factor(s) induced by FluG in *A. nidulans* (D'Souza *et al.*, 2001), although this would require further investigation.

3.5 CONCLUSION

3.5.1 Mannitol is Required for Pathogenicity

The major finding of this investigation is that the phytopathogen *S. nodorum* has a requirement for mannitol in order to complete the process of infection on wheat. The abolition of mannitol synthesis in a double mutant harbouring disruption constructs for mannitol dehydrogenase and mannitol 1-phosphate dehydrogenase resulted in an inability to sporulate *in planta* which could be partially rescued by the addition of exogenous mannitol. This is the first demonstrated role for mannitol in a fungal phytopathogen and suggests that if a means of inhibiting mannitol synthesis could be devised, the polycyclic infection process could be arrested, thus significantly reducing the impact of the disease in a wheat crop.

3.5.2 Enzymatic Cycling of Mannitol is Physiologically Unimportant

A major conclusion arising from this portion of the study was that the mannitol cycle is unlikely to operate in *S. nodorum* as proposed by Hult and Gatenbeck (1978). Instead, the evidence indicates that the metabolism of mannitol in *S. nodorum* occurs by two separate pathways (Figure 3.23).

One of these pathways consists of the dephosphorylation of glycolytic/ gluconeogenic fructose 6-phosphate to fructose by fructose 6-phosphatase (and which can be catalysed in the reverse direction by hexokinase), followed by the reversible reduction of fructose to mannitol by mannitol 2-dehydrogenase. It is also possible that

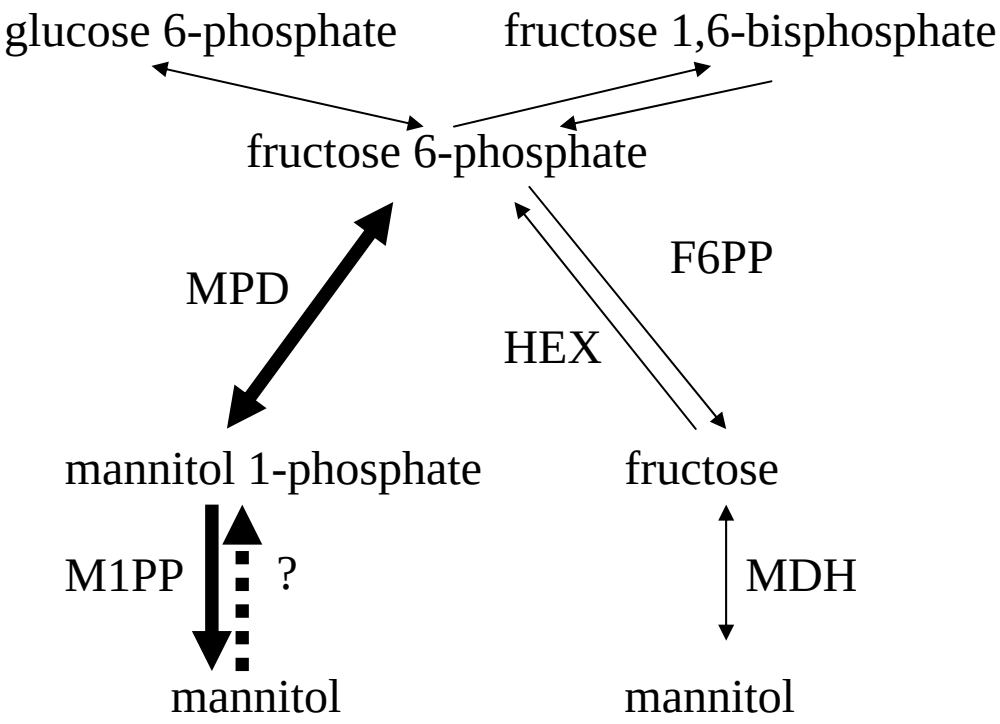

Figure 3.23: The two pathways for mannitol metabolism in *Stagonospora nodorum* showing the enzymes involved in each step including a putative mannitol phosphorylation step catalysed by unknown enzyme(s). The thicker arrows of the mannitol 1-phosphate-mediated pathway indicate that this is the major pathway for mannitol synthesis and catabolism. The glycolytic and gluconeogenic precursors of fructose 6-phosphate are also shown. Abbreviations: MPD = mannitol 1-phosphate dehydrogenase; M1PP = mannitol-1-phosphate phosphatase; Hex = hexokinase; F6PP = fructose-6-phosphate phosphatase; MDH = mannitol dehydrogenase.

host fructose, resulting from the action of fungal/host invertase on host sucrose, could also feed directly into this pathway. Disruption of this pathway produced no phenotype. This indicated that while it may be possible for mannitol to be metabolised in an enzymatic cycle in the wild type, there is no demonstrated physiological requirement for such a cycle.

The other pathway consists of the reversible reduction of fructose 6-phosphate to mannitol 1-phosphate by mannitol 1-phosphate dehydrogenase, followed by the de-phosphorylation of mannitol 1-phosphate to mannitol by mannitol 1-phosphate phosphatase. This final step is also reversible but the exact reaction pathway and enzyme(s) involved are not yet determined.

Of these two demonstrated pathways, it is the mannitol 1-phosphate mediated route which is the major catabolic pathway. It remains to be determined whether mannitol is maintained as a single pool or not. Flux analysis would be required to demonstrate whether the mannitol synthesised by either pathway is temporally or spatially separated in the fungus as has been suggested for *A. niger*.

CHAPTER 4: METABOLOMICS ANALYSIS OF HEALTHY AND DISEASED LEAVES

4.1 INTRODUCTION

4.1.1 The Metabolome and Antimicrobial Metabolites

The metabolome refers to the entire complement of low-molecular-weight metabolites in a biological sample under a given set of conditions. The term was simultaneously coined by Oliver *et al*. (1998) and Tweeddale *et al.* (1998) as a concept complementary to the transcriptome and proteome (the complete set of genes transcribed or proteins synthesised under given conditions respectively). Metabolomics refers to the analysis of the metabolome and encompasses context-sensitive changes in the metabolome which occur with developmental progress, altered growth conditions, and resulting from genetic mutation. Typically the metabolome pertains to a single organism, but investigations of heterogeneous systems are informative, particularly when metabolites are specific to one of the organisms involved. In the study of a pathosystem, metabolomics can be used to identify changes in the metabolite profile which characterise the progress of an infection. Detection of such changes could offer a means for improved understanding of host-pathogen interactions. Provided the metabolites synthesised are sufficiently abundant, it will be possible to detect them by use of an appropriate metabolomics technology. The two main groups of methods currently employed for studies of this nature are spectroscopic methods such as nuclear magnetic resonance (NMR) spectroscopy, and chromatographic methods such as liquid/gas chromatography coupled to mass spectrometry (LC/GC-MS) (Christensen and Nielsen, 1999). The former of these is described more fully in Chapter 5 below and was not considered suitable for use in this part of the study due to its lower sensitivity, requiring

millimolar concentrations of metabolites (Chatham *et al.*, 2003). GC-MS has been used to detect metabolites at nanomolar concentrations, and was the method used for this investigation.

4.1.2 Overview of Technique

4.1.2.1 Gas Chromatography-Mass Spectrometry

Gas chromatography-mass spectrometry (GC-MS) is a popular platform for metabolomics. This is due to well established techniques for sample preparation and analysis, and the availability of two large online public domain metabolite databases, the NIST/EPA/NIH Mass Spectral Library (192,108 compounds) (NIST, Gaithersburg, MD, USA) and the Golm Metabolome Database (Kopka *et al.*, 2005). Other advantages include the ease of use, small sample size, high reproducibility and robustness, and the relatively low costs compared to other spectroscopic and chromatographic methods (Allwood *et al.*, 2008; Garcia *et al.*, 2008).

GC-MS requires analytes to be volatile and thermostable up to 250 ºC. A necessary preparatory step is the derivatisation of the extracted metabolites. This involves a methoximation step to confer thermal stability, and treatment with a silylating compound such as TMS to form volatile trimethylsilyl esters (Halket *et al.*, 2005). The derivatised metabolites are separated by elution through a GC capillary column with a polar stationary phase and using an inert carrier gas (helium in this experiment) as the mobile phase. Retention time (RT) locking to a standard compound (mannitol in the case of this experiment) enables the RTs of metabolites to

be precisely measured. The use of an internal standard not present in the samples (ribitol in the case of this experiment), in conjunction with the sample weights, enables the data to be normalised and permit valid comparisons of metabolite abundances between samples (Solomon *et al.*, 2006b). The mass spectrometer (MS) consists of an ion source, mass analyser, and detection system. As samples are introduced into the MS they are ionised by one of a variety of ion sources including flame ionisation, chemical ionisation or electron ionisation. The latter method was used here and involved bombardment of the gaseous sample molecules with a beam of energetic electrons to generate ions. Mass analysers separate ions based on their mass-to-charge (*m/z*) ratio, and range from the simple and inexpensive linear quadropole (Q) and time-of-flight (TOF) systems, to hybrid Q-TOF and Orbitrap systems, up to the more sensitive and expensive Fourier transform ion cyclotron resonance (FT-ICR) analyser (Dunn, 2008). The first of these systems was used here.

A disadvantage of GC-MS is the requirement for derivatisation. Besides requiring additional sample preparation time, there are a number of artefacts and unexpected by-products which can be generated by the derivatisation step (Little, 1999). It is limited to the detection of low-molecular-weight, volatile compounds. Other MS techniques such as liquid chromatography (LC)-MS are better suited for detection of non-volatile and larger molecular weight metabolites.

4.1.2.2 Principal Components Analysis

Principal Components Analysis (PCA) is a multivariate analysis technique devised by Pearson (1901), and independently developed by Hotelling (1933a;

1933b). The fundamental aim of the technique is to reduce the dimensionality of a data set consisting of a large number of correlated factors (*p*), to a smaller number of uncorrelated factors (*m*), whilst retaining as much of the variation present in the original data set as possible. This is achieved by transforming the data set to a new set of variables, the principal components (PCs), which are numbered in order of decreasing ability to explain the total remaining variation. In a data set in which the variation is non-random, the majority of the variation should ideally be accounted for by the first few principal components. Where *m* is substantially smaller than *p*, the PCs may be amenable to ready interpretation, although this is by no means necessarily the case in all circumstances (Jolliffe, 1986).

4.1.3 Aims of the Study

The aim of this experiment was to use GC-MS, coupled with multivariate analysis, to analyse the metabolites of infected and uninfected tissue of wheat leaves inoculated with *Stagonospora nodorum*. This was designed to compare the two conditions to reveal the presence of lesion-specific metabolites, in addition to any metabolites resulting from a systemic reaction to infection. Mock-inoculated and uninoculated controls were also included.

4.2 MATERIALS AND METHODS

4.2.1 Sample Collection

Benzimidazole agar plates were set up as per a detached leaf assay (Section 3.2.6.1), except that 8 cm sections of leaf were embedded in the agar, following the trimming of 2 cm from the tips of the leaves. A separate plate was prepared for each sample and consisted of 14 leaves per plate. Three biological replicates were prepared for each harvesting time point. Each leaf was inoculated at 2.5 cm from one end of the leaf with 5 μL of a SN15 spore solution consisting of 10^6 spores.mL^{-1} made up in 0.02% Tween 20 in milliQ water. Controls were prepared including leaves mock-inoculated with 0.02% Tween 20 only, and uninoculated leaves. Plates were wrapped in clingfilm and incubated under the standard culture conditions described above (3.2.6.1). Samples were harvested at 0.5, 1, 3, 5 and 8 dpi. At each time point, the portion of the leaves containing the lesion was excised and the 14 lesions per replicate placed in a 1.5 mL eppendorf tube and snap frozen in liquid nitrogen. A second portion from each leaf was harvested from the uninoculated, healthy part of the leaf, with these 14 segments placed in a 1.5 mL eppendorf tube and snap frozen. To reduce the risk of contamination between the diseased and non-diseased leaf portions, the non-diseased portions were harvested before the inoculated/diseased portions. Samples were stored at -80 ºC until ready for metabolite extraction. Samples were defined as:

1. Infected – tissue taken from the site of inoculation with spore solution. In the early stages this comprised a sample of leaf with the attached inoculation droplet whilst for the later stages this comprised the entire lesion formed.

2. Uninfected – healthy tissue taken from a leaf inoculated with spore solution.
3. Mock-inoculated – tissue taken from the site of inoculation with Tween 20 (8 dpi only).
4. Uninoculated – tissue taken from an uninoculated leaf (8 dpi only).

4.2.2 Sample Preparation for GC-MS

Harvested tissue was homogenised in a Retsch MM301 Mixer Mill as described above (Section 2.7.1). Wet weights for each sample were recorded following addition of the homogenate to pre-weighed eppendorf tubes containing 1 mL methanol. Extraction of polar metabolites, derivatisation of samples, and GC-MS spectra acquisition and analysis were performed as described above (Section 2.8).

4.2.3 Data Analysis

Normalisation of metabolite abundances in samples was performed using the wet weight of the samples and the peak area of the ribitol internal standard in each sample. The normalised data set was log-transformed following the addition of "1" to each value in order to account for missing (i.e. zero) values. The transformed data was analysed by Principal Component Analysis using The Unscrambler® v9.8 (Camo Software AS, Oslo). The model was validated using the cross-validation method. Outliers were identified via the Hotelling T^2 ellipse 95% confidence limit. The top 20 variables (metabolites) contributing to the variation accounted for in each of the principal components were subjected to statistical analysis as described previously (Section 2.10).

4.3 RESULTS

4.3.1 GC-MS Peaks

A total of 194 peaks were detected across all samples by GC-MS (Table 4.1). Of these, 99 were identified as a result of comparing fragmentation patterns with the NIST and Golm libraries. These included the ribitol internal standard, and 67 endogenous compounds, some with multiple derivatives, and including methoxyaminated and dephosphorylated versions of some compounds. Two samples (one 1 dpi infected and one 3 dpi uninfected replicate) had to be discarded due to sample spillage in one case, and misinjection by the GC-MS automatic loader in the other.

4.3.2 Principal Components Analysis

Analysis using the Hotelling T^2 ellipse 95% confidence limit resulted in two of the 8 dpi diseased leaf samples being identified as outliers. These were retained in the analysis.

Principal components analysis showed that the first four principal components cumulatively accounted for 76% of the total variance (PC1 = 43%, PC2 = 15%, PC3 = 10%, and PC4 = 8%). The best separation among samples in score plots was achieved by combining PC1 with PC2 (Figure 4.1A).

Table 4.1: Library of retention times (RT) and identities for metabolites detected by GC-MS from healthy and diseased tissue of wheat leaves infected with *Stagonospora nodorum* and harvested at 0.5, 1, 3, 5 and 8 days post infection. Metabolites from negative controls including mock-inoculated and uninoculated leaves are included.

RT	Identity
5.1447	Unknown
5.9261	Unknown
6.0850	Unknown
6.3168	Unknown
8.7803	Unknown
9.1313	L-Valine_2TMS
9.4491	Glycine_3TMS
10.1445	Unknown
10.3167	Glycerol_3TMS
11.1047	Malonic_acid_2TMS
11.7073	Glycine_3TMS
12.1378	L-Serine_2TMS
12.3299	L-Serine
12.3696	Unknown
12.3762	Unknown
12.8000	Phosphoric_acid_3TMS
12.8663	Unknown
12.9789	L-Alanine_3TMS
13.1444	Glyceric_acid_3TMS
13.1643	Unknown
13.4756	Unknown
13.6344	L-Serine_3TMS
13.8663	Unknown
13.9125	Unknown
13.9920	Nicotinic_acid_1TMS
14.0847	L-Threonine_3TMS
14.1642	Succinic_acid_2TMS
14.4092	Unknown
14.8065	Maleic_acid_2TMS
15.2768	Unknown
15.6077	Unknown
16.0284	Threonic_acid-1,4-lactone_2TMS
16.6211	Alanine_3TMS
16.8859	Propanedioic_acid
17.0713	Unknown
17.4556	Unknown
17.5349	L-Aspartic_acid_3TMS
17.7203	4-Aminobutyric_acid_3TMS
17.8197	Malic_acid_3TMS

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Table 4.1: Cont.

RT	Identity
18.0515	Unknown
18.1178	Unknown
18.2701	Unknown
18.3031	Unknown
18.3760	Unknown
18.4686	L-Aspartic_acid_3TMS
18.6209	Unknown
18.8527	Erythronic_acid_4TMS
19.2037	Unknown
19.7136	Xylitol_5TMS
19.9586	Unknown
20.2831	N-Acetylglutamic_acid_2TMS
20.5613	Ribitol_5TMS
20.7136	Arabitol_5TMS
20.8195	L-Glutamic_acid_3TMS
20.9586	Pyroglutamic_acid_2TMS
21.3362	Unknown
21.5347	Galactonic_acid_6TMS
21.7996	Ribonic_acid_5TMS
21.8261	L-Phenylalanine_2TMS
21.8857	Unknown
21.9453	Unknown
22.1970	Unknown
22.4156	Unknown
22.5481	Unknown
22.8327	2-keto-L-Gluconic_acid_5TMS
22.8461	L-Asparagine_3TMS
22.9189	Unknown
23.0447	Unknown
23.1638	Glucaric_acid_6TMS
23.3096	Unknown
23.3361	Ornithine_4TMS
23.6009	Unknown
23.6803	Unknown
23.8063	Unknown
24.3094	Unknown
24.3161	Mannitol_6TMS
24.3427	L-Glycerol-3-phosphate_4TMS
24.3756	D-Quinic_acid_5TMS
24.3823	Unknown
24.4088	Unknown
24.4154	3,1,11,15-Tetramethyl-2-hexadecen-1-ol
24.4551	cis_Aconitic_acid_3TMS
24.5346	Fructose_methoxyamine_5TMS
24.6208	Mannitol_6TMS
24.6405	Unknown

Continued on following page

Table 4.1: Cont.

RT	Identity
24.6538	Fumaric_acid_2TMS
24.6605	Phosphoric_acid_3TMS
24.6671	Unknown
24.6803	Unknown
24.8657	Fructose_methoxyamine_5TMS
24.9717	Unknown
24.9849	Unknown
25.0446	Glucose_methoxyamine_5TMS
25.0843	Citric_acid_4TMS
25.3094	Glucose_methoxyamine_5TMS
25.3690	L-Lysine_4TMS
25.4021	Unknown
25.4950	Unknown
25.5147	Isocitric_acid_4TMS
25.6140	cis-Aconitic_acid_3TMS
25.8326	Xylitol_5TMS
25.9650	Glyceric_acid-3-phosphate_4TMS
26.0511	Unknown
26.1835	Unknown
26.2696	Unknown
26.3226	Unknown
26.3756	L-Asparagine_4TMS
26.4220	Unknown
26.5082	Unknown
26.5744	1-Ethylglucopyranoside_4TMS
26.6605	Glucopyranose_5TMS
26.7663	Glucose_5TMS
26.9186	Unknown
27.0908	Galactonic_acid_6TMS
27.1835	Unknown
27.3160	Glucaric_acid_6TMS
27.5411	myo_Inositol_6TMS
27.6008	L-Aspartic_acid_3TMS
27.6868	Galactinol_9TMS
27.6935	Unknown
27.7796	L-Tyrosine_3TMS
27.8127	Gulose_5TMS
28.0444	Unknown
28.1571	β-1-Galactopyranoside
28.2100	Unknown
28.3556	Galactose_methoxyamine_5TMS
28.7132	Glucaric_acid_6TMS
28.7861	L-Aspartic_acid_3TMS
28.8125	Sedoheptulose_6TMS
28.8656	Hexadecanoic_acid_1TMS
29.0642	1-Methyl-β-D-galactopyranoside_4TMS

Continued on following page

Table 4.1: Cont.

RT	Identity
29.3961	Unknown
29.3027	Unknown
29.4086	Unknown
29.9053	Unknown
30.5344	2-O-Glycerol-β-D-galactopyranoside_6TMS
30.5411	Unknown
30.6801	Unknown
30.7794	2-O-Glycerol-β-D-galactopyranoside_6TMS
31.0112	1-Methyl-β-D-galactopyranoside_4TMS
31.3489	Unknown
31.7793	1-Methyl-β-D-galactopyranoside_4TMS
32.0641	Unknown
32.1701	Unknown
32.2893	Octadecanoic_acid_1TMS
32.4548	Unknown
32.4614	Ribitol_5TMS
32.8986	Unknown
33.0707	α-Linolenic_acid
33.4415	Unknown
34.0243	Glucose-6-phosphate_methoxyamine_6TMS
34.2429	L-Tryptophan_3TMS
34.4350	myo-Inositol-2-phosphate_7TMS
34.6070	D-Glucuronic_acid_5TMS
35.2760	Glucopyranose_5TMS
35.5542	Unknown
35.5739	Unknown
35.8387	Unknown
36.1568	Unknown
36.5342	Unknown
36.7262	Maltose_methoxyamine_8TMS
37.0043	Sucrose_8TMS
37.0838	Unknown
37.2228	Unknown
37.3751	Galactinol_9TMS
38.4479	Trehalose_8TMS
40.1300	Unknown
40.6599	myo_Inositol_6TMS
40.6796	Unknown
41.0704	Unknown
41.1963	Unknown
41.3418	Galactinol_9TMS
41.7723	Unknown
41.7791	Unknown
41.9115	Unknown
42.0438	Unknown
42.6928	Unknown

Continued on following page

Table 4.1: Cont.

RT	Identity
42.8451	Unknown
43.2557	Unknown
43.6795	Melibiose_8TMS
44.3022	Octacosanol_1TMS
44.3352	Lanost-8-ene-3-β,7-α-diol,3-acetate
44.5537	D-Glucuronic_acid_5TMS
46.1497	Sucrose_8TMS
47.6795	Unknown
48.0767	Unknown
48.6793	β-Sitosterol_1TMS
51.3017	Unknown
52.0368	Gulose_5TMS
53.8777	Sucrose_8TMS
53.9308	Unknown
54.5996	Unknown
54.6593	Unknown
54.6726	Sucrose_8TMS

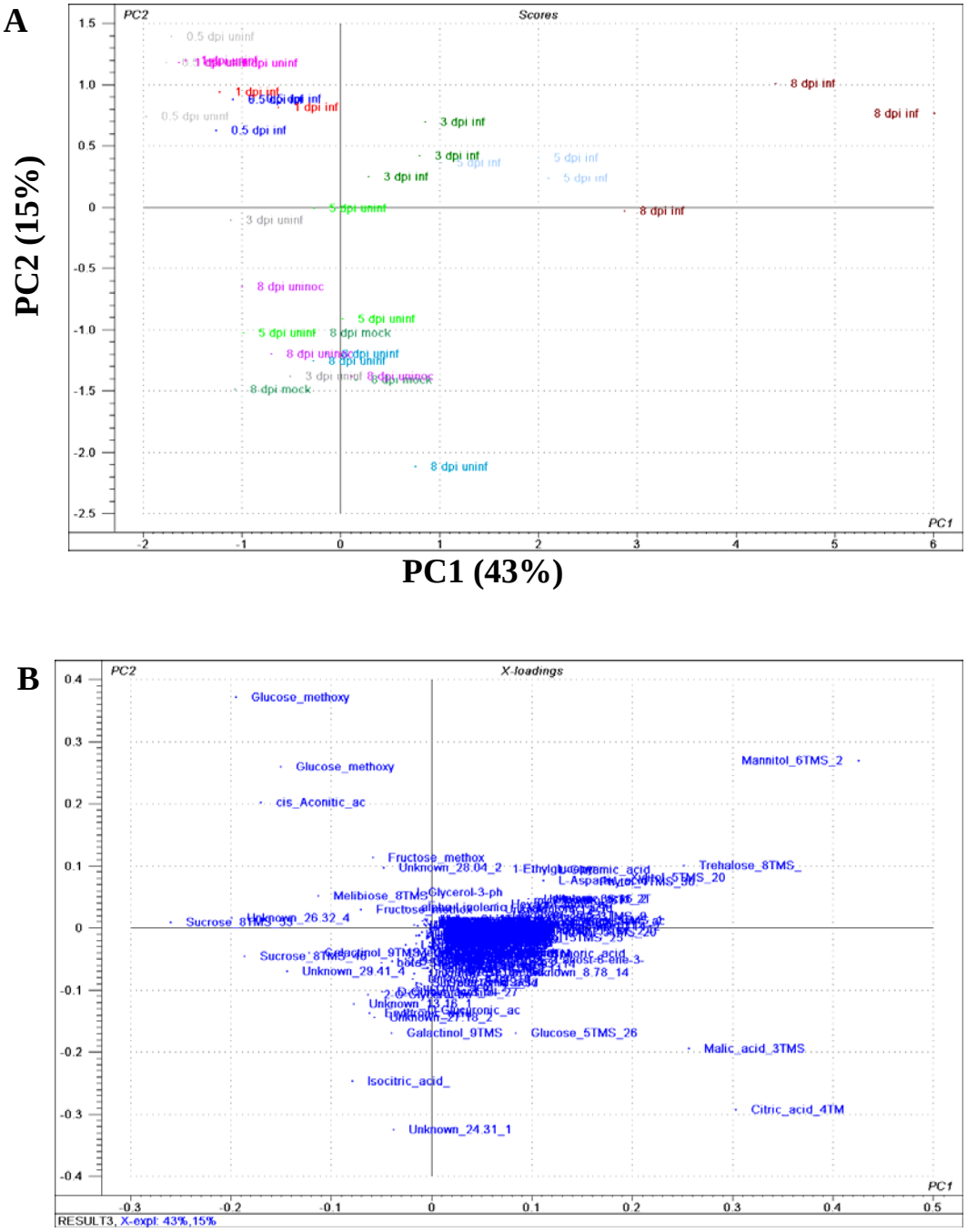


Figure 4.1: Principal components analysis (PCA) score plot **(A)** and loading plot **(B)** for PC1 versus PC2 from a PCA of polar metabolites processed by GC-MS. Samples consisted of tissue harvested at 0.5, 1, 3, 5 and 8 days post inoculation (dpi) from a detached leaf assay. Tissue was harvested from diseased (inf) and healthy (noninf) tissue of leaves inoculated with *Stagonospora nodorum* spores. Control leaves were either mock inoculated (mock) or uninoculated (uninoc) (both 8 dpi only).

Considering the principal components individually, PC1 exhibited differentiation between the samples both in terms of time to sample harvest, and infection. While the separation in the uninfected samples was not large, there was nevertheless an observable continuum with the early stage samples at one end and the late stage samples at the other. All diseased tissue samples from 3 dpi and later had positive scores with respect to PC1 and with a positive trend between the score and time to harvest. All remaining samples had negative scores with respect to PC1 with the exception of one 5 dpi uninfected sample and three 8 dpi samples (one uninfected, one mock inoculated and one uninoculated). Three of these four had low positive scores, while the 8 dpi uninfected sample had a score which placed it among the 3 dpi infected samples. There was a trend observed whereby score increased with time to harvest. Loadings for each of the top 20 variables (metabolites) contributing to PC1 are shown (Figure 4.2A).

The samples were clustered into three groups by PC2. The early stage uninfected samples up to and including 1 dpi formed one cluster all with positive scores, while the remaining uninfected, mock-inoculated and uninoculated samples formed a second cluster all with negative scores. Apart from a couple of interpolated samples, all of the infected samples formed a discrete cluster between these two. Loadings for each of the top 20 metabolites contributing to PC2 are shown (Figure 4.2B).

The PCA loading plot for PC1 versus PC2 (Figure 4.1B) gave emphasis to some of the factors separating the samples. Mannitol and trehalose were key metabolites defining the infected samples particularly in the later stages of infection,

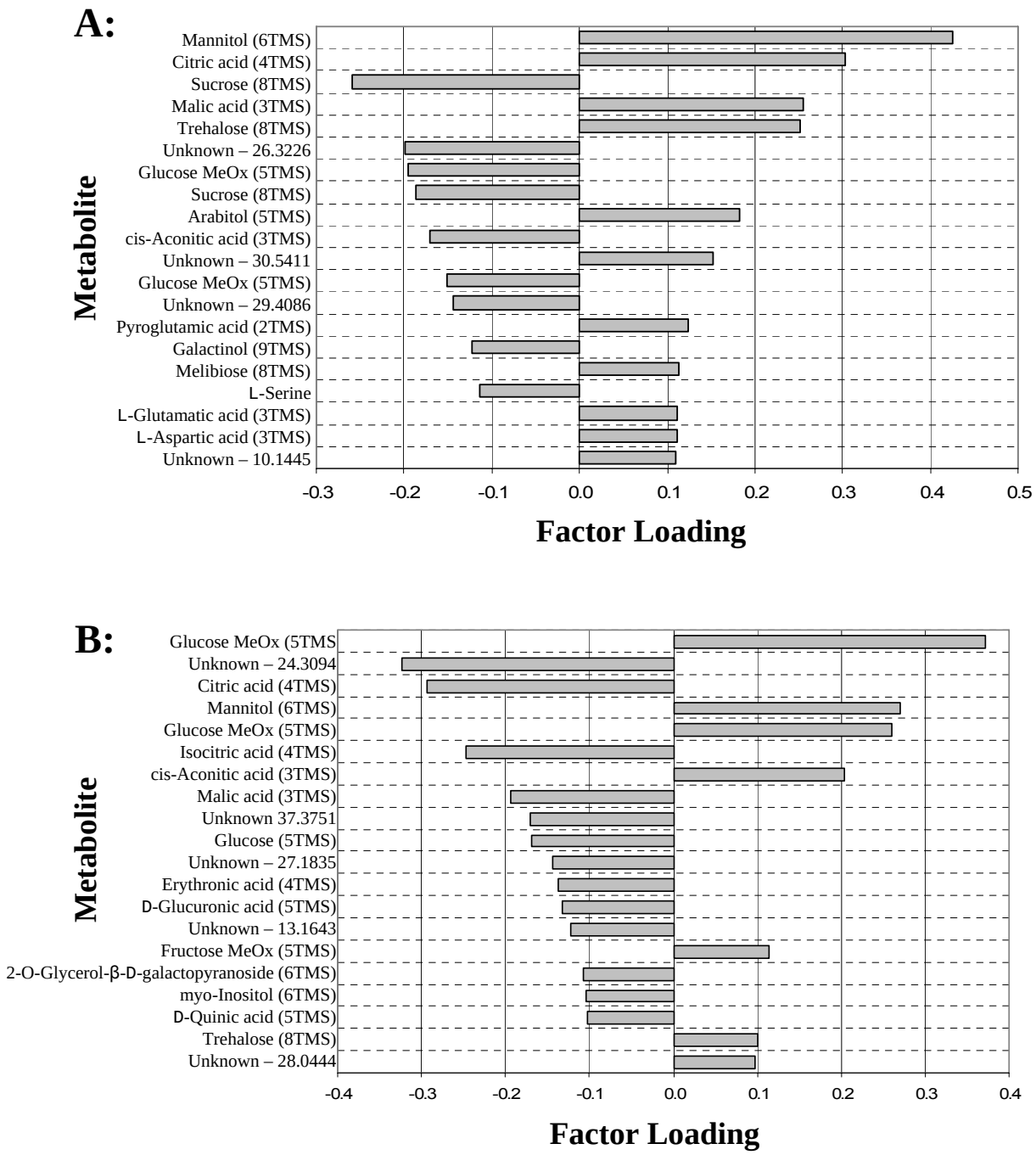


Figure 4.2: The top 20 variables (metabolites) contributing to the variation accounted for by PC1 **(A)** and PC2 **(B)** in a PCA of healthy and *Stagonospora nodorum*-infected wheat leaf tissue. Metabolites are arranged on the Y-axis in order of magnitude of factor loading (regardless of sign). Unidentified metabolites are shown with their retention time. Note that the scales on the X-axis are not identical.

while sucrose and glucose were defining features of the uninfected/mock inoculated/uninoculated and early stage infection samples. The early stage samples of all treatments were associated with cis-aconitate. They also had high levels of glucose, although other derivatives of glucose were also found in later samples. The later stage uninfected/mock inoculated/uninoculated samples were also characterised by isocitrate, and the unknown compound with a RT of 24.3094. Malate and citrate were both negatively correlated with the early samples and positively correlated with the later samples, particularly where infection was present. Out of the top 20 metabolites for each principal components, seven metabolites contributed to both PC1 and PC2 (malate, mannitol, cis-aconitic acid, glucose methoxyamine (both derivatives), citrate, and trehalose).

4.3.3 Statistical Analysis of Metabolites Identified by PCA

There were 33 unique GC-MS peaks in the top 40 variables contributing to PC1 and PC2 as determined by their factor loadings. When the normalised GC-MS data was analysed for each of these, 9 exhibited no statistically significant differences between samples. The remaining 24 exhibited a number of expression profiles.

4.3.3.1 Metabolites Present Only in Diseased Samples

There were three metabolites present only in infected tissue (Figure 4.3). The presence of these metabolites was only statistically significant at 8 dpi, although arabitol and mannitol were present at earlier stages of infection and displayed a trend whereby they increased with time of infection. Trehalose was only present at 8 dpi.

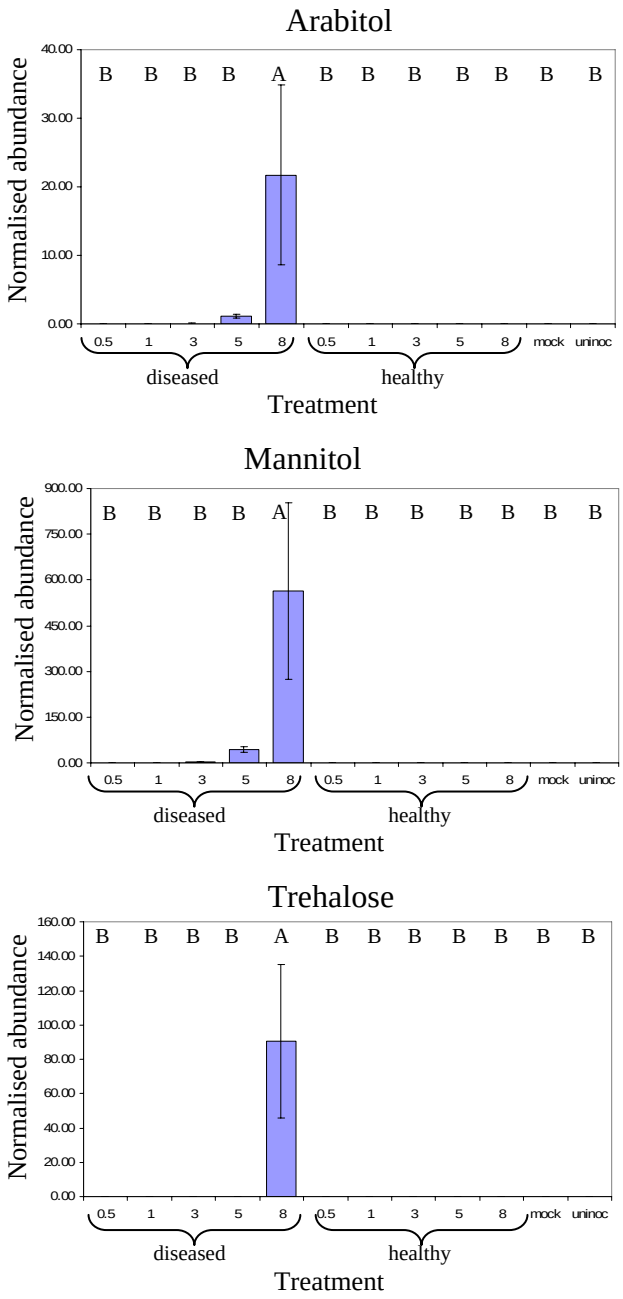


Figure 4.3: Mean normalised abundance (±SE) for metabolites present only in diseased tissue. Treatments consisted of diseased and healthy tissue sampled at 0, 1, 3, 5 and 8 dpi from wheat leaves inoculated with *Stagonospora nodorum*. Mock-inoculated and uninoculated controls were harvested at 8 days. GC-MS peak areas were normalised by division using the peak area of an internal standard (ribitol) followed by division by the sample wet weight. Statistical significance was calculated for each group using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. N=3 for all treatments except 1 dpi infected and 3 dpi uninfected (N=2).

4.3.3.2 Metabolites Increasing with Time of Infection in Diseased Samples

Six metabolites were present in all treatments, but were significantly higher in later stage diseased samples (Figure 4.4). None of these metabolites exhibited any significant difference in abundance within the healthy tissue samples with the exception of glucose. In this case the 8 dpi uninfected samples had significantly more glucose than the 0.5 dpi samples.

4.3.3.3 Metabolites Significantly Higher in Healthy Tissue than Diseased Tissue

Six metabolites were significantly more abundant in some healthy samples than in any diseased samples (Figure 4.5). In the case of the two glucose methoxyamine derivatives, sucrose, and an unknown (RT 28.0444) the compound was highest in the early stage uninfected (0.5 to 1 dpi) samples. In the case of D-glucuronic acid and isocitric acid, the compound was highest in the late stage (3 to 8 dpi) samples.

4.3.3.4 Metabolites Significantly Lower in Late Stage Diseased Tissue

Nine metabolites were present in both diseased and healthy tissue, but were significantly lower or absent in 5 dpi and/or 8 dpi infected samples (Figures 4.6 and 4.7). All were compounds with low abundances. Six were compounds with no match in the MS databases. One unknown with an RT of 24.3094 was not identified in any diseased sample.

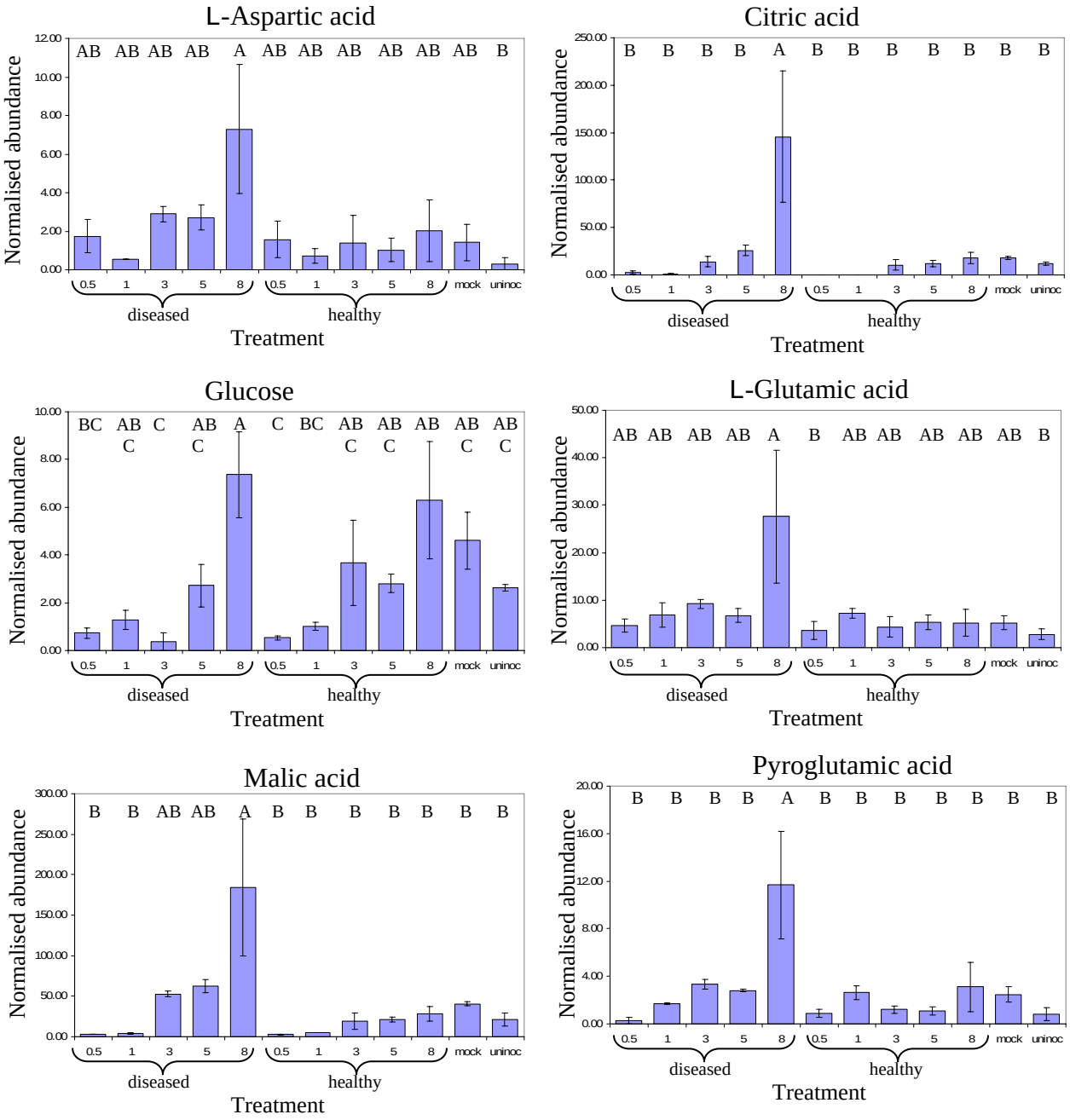


Figure 4.4: Mean normalised abundance (±SE) for metabolites significantly higher in later stage infected tissue. Treatments consisted of diseased and healthy tissue sampled at 0, 1, 3, 5 and 8 dpi from wheat leaves inoculated with *Stagonospora nodorum*. Mock-inoculated and uninoculated controls were harvested at 8 days. GC-MS peak areas were normalised by division using the peak area of an internal standard (ribitol) followed by division by the sample wet weight. Statistical significance was calculated for each group using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. N=3 for all treatments except 1 dpi infected and 3 dpi uninfected (N=2).

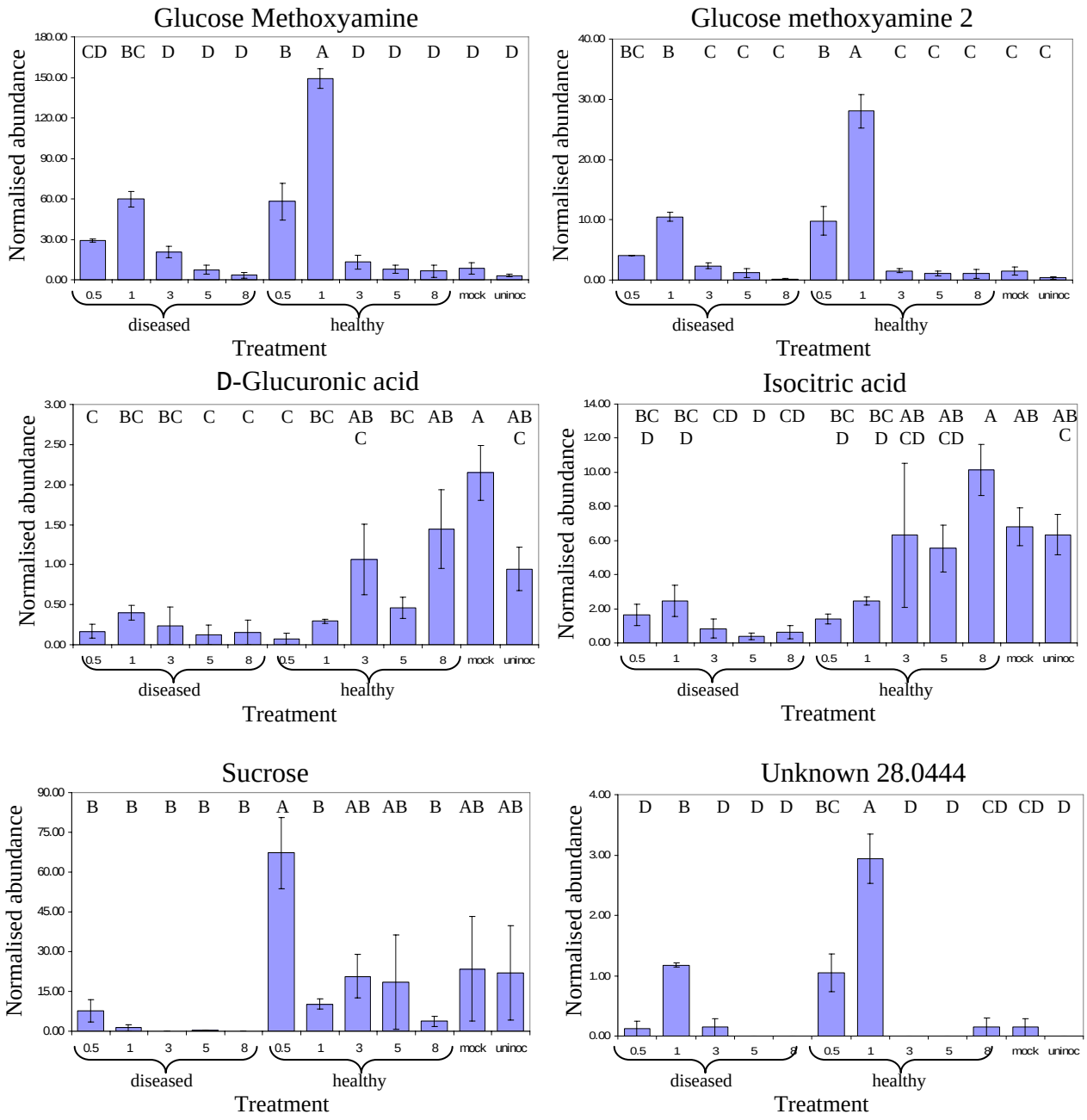


Figure 4.5: Mean normalised abundance (±SE) for metabolites significantly higher in healthy tissue. Treatments consisted of diseased and healthy tissue sampled at 0, 1, 3, 5 and 8 dpi from wheat leaves inoculated with *Stagonospora nodorum*. Mock-inoculated and uninoculated controls were harvested at 8 days. GC-MS peak areas were normalised by division using the peak area of an internal standard (ribitol) followed by division by the sample wet weight. Statistical significance was calculated for each group using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. N=3 for all treatments except 1 dpi infected and 3 dpi uninfected (N=2).

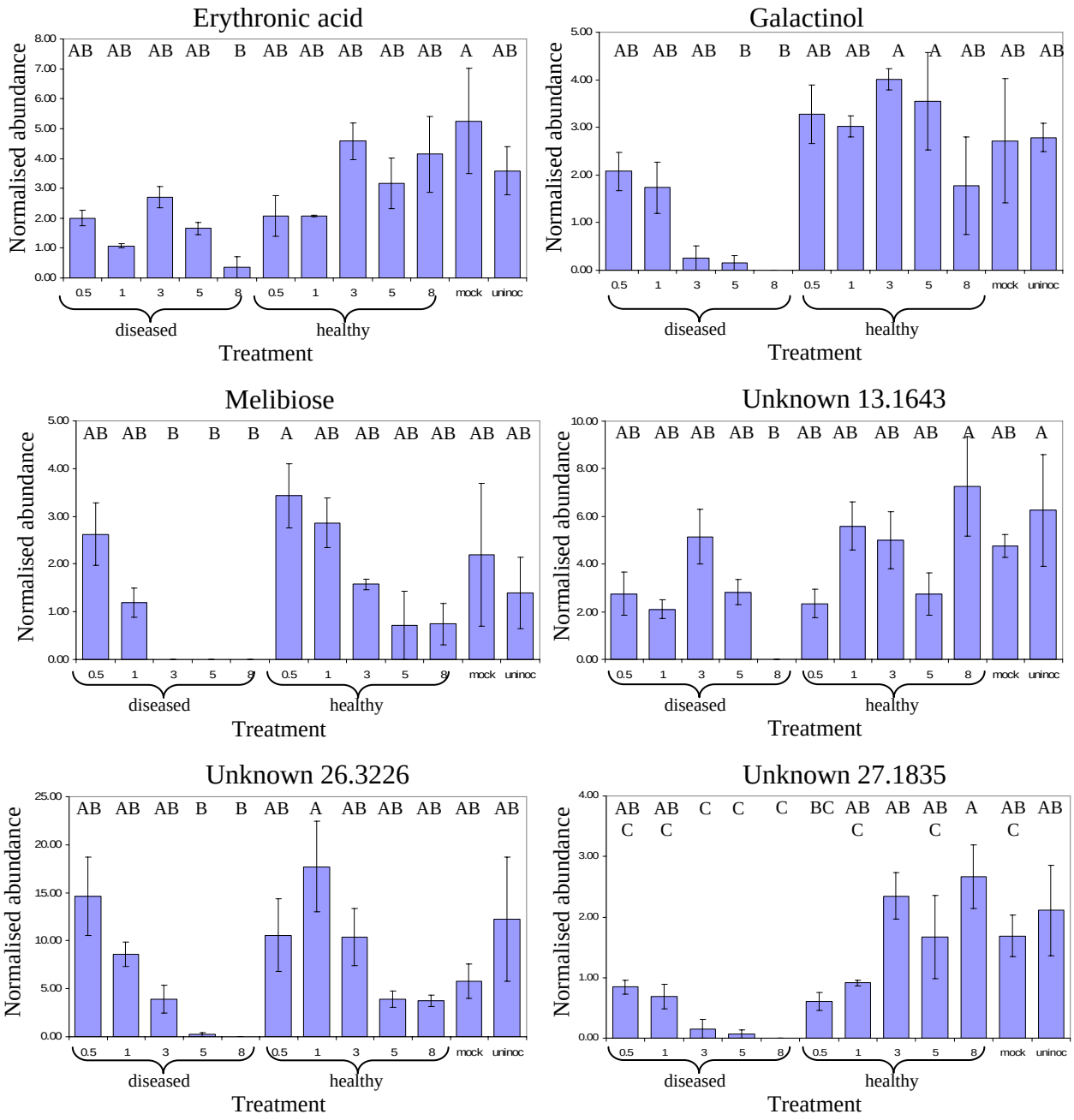


Figure 4.6: Mean normalised abundance (±SE) for metabolites significantly lower in late stage diseased tissue. Treatments consisted of diseased and healthy tissue sampled at 0, 1, 3, 5 and 8 dpi from wheat leaves inoculated with *Stagonospora nodorum*. Mock-inoculated and uninoculated controls were harvested at 8 days. GC-MS peak areas were normalised by division using the peak area of an internal standard (ribitol) followed by division by the sample wet weight. Statistical significance was calculated for each group using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. N=3 for all treatments except 1 dpi infected and 3 dpi uninfected (N=2).

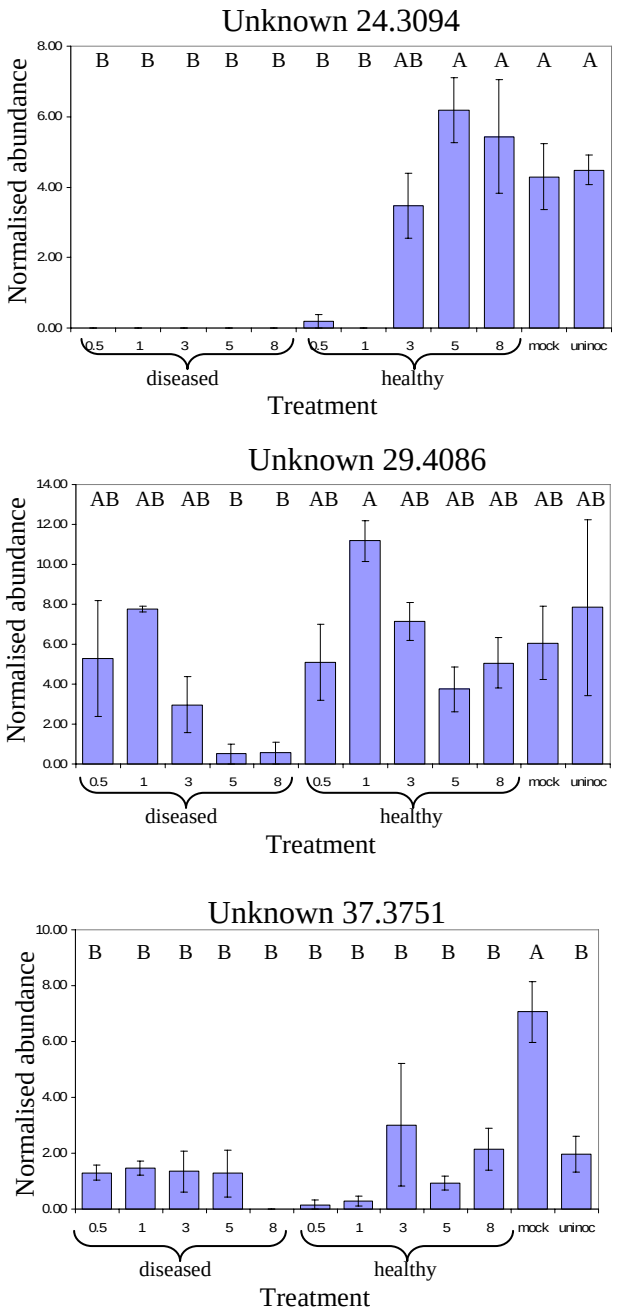


Figure 4.7: Mean normalised abundance (±SE) for metabolites significantly lower in late stage diseased tissue. Treatments consisted of diseased and healthy tissue sampled at 0, 1, 3, 5 and 8 dpi from wheat leaves inoculated with *Stagonospora nodorum*. Mock-inoculated and uninoculated controls were harvested at 8 days. GC-MS peak areas were normalised by division using the peak area of an internal standard (ribitol) followed by division by the sample wet weight. Statistical significance was calculated for each group using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. N=3 for all treatments except 1 dpi infected and 3 dpi uninfected (N=2).

4.4 DISCUSSION

A non-targeted GC-MS approach was used to examine the polar metabolomes of healthy and diseased wheat leaf tissue from infected leaves, compared to tissue from mock-inoculated and uninoculated leaves. PCA was used to identify metabolites which contributed to the principal components explaining most of the variation in the data set. Metabolites of interest were subjected to statistical analysis to determine those whose abundance was significantly different between treatments.

4.4.1 Compounds Associated with Infected Tissue Only

The statistically significant difference represented by the presence of mannitol, trehalose and arabitol in the 8 dpi infected samples is the most likely reason for two of these replicates being identified as outliers. Since the changes in abundance of these compounds is of biological significance, they were not excluded from the data set.

4.4.1.1 Mannitol

Mannitol levels rose as the disease progressed, although this increase was only statistically significant in the 8 dpi samples. Mannitol was not detected in the healthy tissue samples. This compound was demonstrated as being required for sporulation in Chapter 3 of this study. The concentration of mannitol was previously shown to increase dramatically with infection in this pathosystem (Lowe, 2006). Mannitol is the most abundant polyol found in fungi, and has also been described in over 50 plant families (Lewis and Smith, 1967), but has not been reported in wheat. In a study

involving the ectopic expression of the mannitol-1-phosphate dehydrogenase (*mtlD*) gene of *Escherichia coli* in wheat, there was no mannitol detected in –mtlD plants, while +mtlD plants constitutively produced mannitol (Abebe *et al.*, 2003). Mannitol has previously been suggested as a fungal-specific compound in the *Cladosporium fulvum*-tomato and *Sclerotinia sclerotiorum*-sunflower pathosystems based on the presence of the compound in infected, but not uninfected tissue (Clark *et al.*, 2003; Jobic *et al.*, 2007). It is likely that mannitol is a fungal-specific compound in the *S. nodorum*-wheat pathosystem.

4.4.1.2 Trehalose

Trehalose is a glucose dimer which is widely distributed among bacteria, fungi, insects, invertebrates and plants (Elbein *et al.*, 2003). While trehalose accumulation was noted for many microorganisms including phytopathogenic fungi, it was not considered to occur widely in plants until the unexpected discovery in *Arabidopsis thaliana* of a plethora of trehalose biosynthesis genes (Leyman *et al.*, 2001). Prior to this trehalose had only been associated with the "resurrection" plants *Selaginella lepidophylla* and *Myrothamnus flabellifolia* under conditions of water stress (Müller *et al.*, 2001). Putative trehalose biosynthetic genes have since been identified in wheat transcripts/ESTs induced by abiotic stress (Ramalingam *et al.*, 2006; Mohammadi *et al.*, 2007), and trehalose and enzyme activities corresponding to trehalose biosynthetic enzymes were reported from wheat subjected to salt and water stress (El-Bashiti *et al.*, 2005). The generally low levels of trehalose reported from higher plants has been suggested as being due to the ubiquitous production of the trehalose-degrading enzyme trehalase (El-Bashiti *et al.*, 2005). The observation here

of trehalose only in the 8 dpi infected samples accords with a previous report that trehalose concentration dramatically increased in the *S. nodorum*-wheat pathosystem in concert with pycnidia production (Lowe, 2006). Trehalose was considered to be a fungal-specific compound in the *S. sclerotiorum*-sunflower pathosystem since it was detected in fungal extracts and infected tissue, but not in uninfected plant tissue (Jobic *et al.*, 2007). Disruption of trehalose 6-phosphate synthase (*Tps1*) in *S. nodorum* reduced trehalose levels in infected tissue to 1% of wild type levels with markedly reduced sporulation, but little effect on the ability of the mutants to cause lesions (Lowe, 2006). Similarly, traces of trehalose were still detectable in *tps1* mutants in *M. grisea* (Foster *et al.*, 2003) and *Botrytis cinerea* (Doehlemann *et al.*, 2006). These reports suggest that while trehalose production by the host in response to pathogen attack cannot be ruled out, it would appear that the nearly all of the trehalose observed in infected tissue is likely to be of fungal origin.

4.4.1.3 L-Arabitol

Levels of the pentitol L-arabitol rose from 3 dpi on as the disease progressed, although this increase was only statistically significant in the 8 dpi diseased tissue samples. This compound was previously reported as having a role in osmotolerance in *S. nodorum* (Lowe *et al.*, 2008), *Magnaporthe grisea* (Dixon *et al.*, 1999) and *C. fulvum* (Clark *et al.*, 2003). In the ^{13}C NMR study presented in Chapter 5, arabitol was the second most abundant metabolite detected in *in vitro* cultures of the wild type. Pentitols other than ribitol have rarely been reported from plants (Lewis and Smith, 1967). L-arabitol was reported to be converted to L-ribulose in tobacco, pea and wheat, although L-arabitol was not considered to be a natural substrate in any of these

plants (Kocourek *et al.*, 1964). L-Arabitol was considered to be a fungal-specific compound in the *C. fulvum*-tomato pathosystem since it was detected in infected tissue, but not in uninfected plant tissue (Clark *et al.*, 2003). While it is most likely to be fungal-specific in the pathosystem investigated in this study, this question could be resolved by abolishing L-arabitol production in the fungus. However, mutants in which genes for L-arabitol dehydrogenase (*Abd1*) and/or L-xylitol dehydrogenase (*Xdh1*) were disrupted, were still able to produce basal levels of L-arabitol, and an additional putative L-arabitol synthesis gene was identified in the *S. nodorum* genome (Lowe *et al.*, 2008). These mutants were all fully pathogenic. Targeted gene replacement of the osmosensory MAP kinase-encoding gene *OSM1* in *M. grisea* resulted in drastically reduced arabitol production and sensitivity to osmotic stress, but had no effect on pathogenicity (Dixon *et al.*, 1999). Thus, while this compound was seen here to increase significantly in the late stages of infection, there was no evidence that it is required for pathogenicity.

4.4.2 Plant Specific Compounds

Sucrose was significantly higher in the 0.5 dpi healthy tissue samples than in any of the diseased samples. This is consistent with the conversion of this compound to its glucose and fructose moieties by host and/or fungal invertase in infected tissue, and with the fungus acting as a carbon sink. Sucrose was considered to be a plant-specific metabolite in this pathosystem. This was based on the fact that:

1. Sucrose was present in all healthy tissue samples.
2. Sucrose was not detected in the ^{13}C NMR study of the fungus described in Chapter 5 below.

3. There has been no report of a sucrose synthase in any fungal species and a BLAST of the *S. nodorum* genome sequence using the *Tuber solanum* sucrose synthase protein sequence (Accession #P10691) produced no hits.

Melibiose is a disaccharide of glucose and galactose and is a hydrolysation product of the plant trisaccharide raffinose (Hepworth, 1924). Galactinol is formed from UDP-galactose and *myo*-inositol and has no known function other than as a precursor for the formation of the raffinose family oligosaccharides (Zhao *et al.*, 2004). The observed trend whereby these metabolites decreased with time of infection to their statistically significant absence in the 8 dpi samples is consistent with their representing a plant carbon resource which was being consumed by the pathogen.

4.4.3 Miscellaneous Metabolites

The majority of the remaining compounds which exhibited a significant difference between treatments, were detected in all samples. The exception to this observation was the unknown with an RT of 24.3094 which was strongly associated with the later stage non-diseased samples. This unknown was not particularly abundant in any sample and it would appear that its proximity to the RT of mannitol resulted in the presence of this unknown compound in the infected samples being obscured. An extracted ion chromatogram for this unknown suggested that it was also present in at least some of the infected samples, but the abundance was so low that its detection was not certain. It obtained very poor matches from the metabolite databases with the best score being for sorbitol. If this unknown represents an

authentic metabolite, it would appear to be a sugar or sugar alcohol of uncertain identity.

The remaining metabolites consisted of amino acids and amino acid derivatives (aspartate, glutamate, pyroglutamate), tricarboxylic acid (TCA) cycle intermediates (citrate, isocitrate, malate), glucose/glucose derivatives, organic acids (D-glucuronic acid, erythronic acid) and seven unknowns. None of the identified compounds were specific to either the host or the pathogen. There are a number of interpretations that could be made in terms of the observed changes in abundance. For instance, compounds which appeared to decrease with time of infection could be represented as

1. plant metabolites which were being consumed by the pathogen
2. fungal metabolites which were required during the early stages of infection but not at the later stages
3. a combination of the above

Without a means of discriminating between the two potential sources of these metabolites, it was not possible to draw a conclusion regarding the biological significance of changes in their abundances during the infection process. Organic acids have previously been shown to be pathogenicity factors/toxins in some pathosystems e.g. fumaric acid and oxalic acid (Scheffer, 1983). It is therefore of interest that several of these have shown up as being significantly more abundant in the 8 dpi infected samples. The fact that these tissues were necrotic, the significant reduction in or absence of major plant oligosaccharides, and the significant increase in fungal-specific metabolites, is strongly suggestive that the metabolites present in these

tissues would either be of fungal origin, or have been maintained by the fungus rather than degraded. It is likely then that the metabolites falling into this category warrant further investigation by targeted gene disruption in the fungus.

4.4.4 No Evidence Found For an Induced Defence Response in the S. nodorum*-Wheat Pathosystem*

Many pathosystems are characterised by the production by the host of antimicrobial metabolites such as the constitutively synthesised phytoanticipans, and the pathogen-attack-induced phytoalexins (VanEtten *et al.*, 1994). Previous studies on wheat phytoalexins have focused on the larger, non-polar secondary metabolites and were investigated by LC-MS and HPLC (Hashimoto *et al.*, 1995; Rémus-Borel *et al.*, 2005; Rémus-Borel *et al.*, 2006). A number of polar phytoalexins have previously been described in other plants including resveratrol in tomato (Ragab *et al.*, 2006), rishitin and lubimin in potato (Fanelli *et al.*, 1992), β-ionone, geranylacetone and terpinyl acetate in cantaloupe (Lamikanra *et al.*, 2002), momilactone A in rice (Atawong *et al.*, 2002), and galactinol in cucumber (Kim *et al.*, 2008). Such a compound would have featured as a significant difference between the mock-inoculated/uninoculated controls and the healthy tissue from the infected leaves. There was no such compound observed. While galactinol was seen to be significantly higher in the later stage healthy versus diseased tissue from infected leaves, it was not significantly higher than the non-infected control. It may be that *S. nodorum* does not elicit a defence response from the host, or that the levels of any such response were below the detection limits of the method used. Since the wheat plants were not grown

under conditions of absolute sterility, it is also possible that endogenous micro-organisms may already have primed any inducible defence response.

4.5 CONCLUSION

An undirected GC-MS metabolomics analysis of healthy and diseased tissue from wheat leaves infected with *S. nodorum* was undertaken. PCA of the data set highlighted metabolites which contributed to the principal components explaining the variation between the treatments. Statistical analysis of these metabolites showed that the fungus-specific or -associated metabolites mannitol, trehalose and arabitol were significantly higher in the later stage infected samples. The plant-specific metabolites sucrose, galactinol and melibiose were absent in the later stage infected samples. A number of amino acids, organic acids, TCA cycle intermediates and unknown metabolites showed some significant changes in abundance in healthy versus infected tissue and may warrant further investigation. It was not possible to conclusively determine the organism of origin of these latter compounds in the infected tissue.

There were no metabolites which differentiated healthy tissue from pathogen-inoculated versus non-inoculated leaves so as to suggest an inducible host defence response in the former.

CHAPTER 5: ^{13}C-NMR INVESTIGATION OF MANNITOL METABOLISM IN *Stagonospora nodorum*

5.1 INTRODUCTION

5.1.1 Overview of Technique

The technique of nuclear magnetic resonance (NMR) spectroscopy is founded on the existence of a magnetic moment in the nuclei of atoms with an odd mass number, or an even mass number but odd atomic number, with the result that these behave like spinning magnetic bodies (Stothers, 1972). When a strong external magnetic field, H_0, is applied to such nuclei, their magnetic fields align either parallel or antiparallel to H_0. Upon irradiation with radiofrequency energy of the correct frequency, the lower-energy-state, parallel-aligned nuclei absorb energy and spin-flip to the higher energy state, and are said to be in resonance with the applied radiation (McMurry, 1984). The spectra produced by modern NMR spectrometers plot the effective magnetic field strength applied to nuclei against their intensity of absorption of energy (Ratcliffe, 1996).

The measurement of the nuclear magnetic moment was first achieved by use of the molecular beam resonance method (Rabi *et al.*, 1938; Rabi *et al.*, 1939) involving the changes in orientation of nuclear spin exhibited by molecular beams in a strong, externally applied magnetic field and in a high vacuum. It was subsequently and simultaneously demonstrated by refinements in the technique, that NMR spectroscopy could be applied to bulk solid (Purcell *et al.*, 1946) and liquid samples (Bloch, 1946; Bloch *et al.*, 1946) and with improvements in both sensitivity and precision. Purcell and Bloch were awarded the Nobel Prize for physics in 1952 for their work on NMR.

An unexpected and surprising observation was next made, that the frequency of resonance of ^{19}F and ^{14}N nuclei was dependent upon the chemical compound in which they were contained (Dickinson, 1950; Proctor and Yu, 1950). The term "chemical shift" was coined in order to describe the observed differences in radiofrequency required to bring isotopic nuclei in different chemical environments into resonance (Arnold *et al.*, 1951).

The progress of ^{13}C-NMR spectroscopy was initially limited by the high detection threshold of the spectrometers, and the low natural abundance (1.1%) of the ^{13}C isotope (Aubert *et al.*, 1996a). Improvements in instrument sensitivity and computer techniques have led to NMR being used extensively for structural analysis of novel compounds, and for metabolite profiling (McMurry, 1984). When used in conjunction with growth on ^{13}C-enriched substrates, it provides a powerful tool for following the metabolism of that substrate, for illustrating differences in metabolism on different substrates and between different strains and species, and for elucidating pathways of carbon metabolism (Jeffrey *et al.*, 1991; Ratcliffe, 1996). In conjunction with other techniques of magnetic resonance, it currently has a wide range of applications in physiology, biology, chemistry, pharmacy and medicine (Shulman and Rothman, 2005; Webb, 2006).

5.1.2 Advantages and Disadvantages of NMR

One of the advantages of NMR compared to other metabolome characterisation methods such as GC-MS, is that no chemical alteration or isolation of

the compounds is required, requiring relatively simple sample preparation and reducing the incidence of experimental artefacts (Yoshida *et al.*, 1984; Pfeffer and Shachar-Hill, 1996). The technique is also non-destructive (Yoshida *et al.*, 1984) , allowing spectra of different nuclei to be acquired for the same sample, and permitting the sample to be subsequently analysed by a different technique (Last *et al.*, 2007). ^{13}C-NMR spectra can be acquired from intact mycelium or from mycelial extracts, and the two procedures have been shown to deliver equivalent spectra (Martin *et al.*, 1985; Ratcliffe, 1996). The use of ^{13}C-labelled substrates turns the low natural abundance of ^{13}C to advantage, since it allows the identification of multiply-labelled metabolites and the quantitation of the label distributed in the spectrum (Ratcliffe, 1996; Pfeffer *et al.*, 2001).

Its main disadvantage is that it is relatively insensitive technique requiring milligram amounts of sample with metabolites required to be present at millimolar concentrations in order to be detected (Pfeffer *et al.*, 2001; Chatham *et al.*, 2003). There are some issues involved with comparisons between spectra on the basis of chemical shifts (Wishart and Sykes, 1994; Wishart and Case, 2001) which are discussed below. The quantitation of the proportion of ^{13}C accruing to different resonances in a spectrum, and comparing this between spectra, can also be problematic. There are a number of approaches to handling this issue and these are also discussed below.

5.1.3 ^{13}C-NMR Studies in Filamentous Fungi

^{13}C-NMR studies have been conducted on a range of filamentous fungi including ectomycorrhizal ascomycetes and basidiomycetes, saprophytes and phytopathogens. The purposes of these studies have ranged from providing baseline information for fungicidal mechanism-of-action studies (Forgue *et al.*, 2006), to furthering the understanding of carbon assimilation and cycling pathways in fungi (Martin *et al.*, 1984; Dijkema *et al.*, 1985; Dijkema and Visser, 1987; Thomas and Baxter, 1987; Martin *et al.*, 1988; Ramstedt *et al.*, 1989; Peksel *et al.*, 2002; Rangel-Castro *et al.*, 2002), including how these pathways are affected in mycorrhizal fungi and phytopathogens under free living versus host-associated conditions (Shachar-Hill *et al.*, 1995; Martin *et al.*, 1998; Bago *et al.*, 1999; Jobic *et al.*, 2007). Key conclusions from these studies have been that mannitol is amongst the most abundant soluble metabolites (Yoshida *et al.*, 1984; Dijkema *et al.*, 1985; Martin *et al.*, 1985; Dijkema and Visser, 1987; Ramstedt *et al.*, 1989; Peksel *et al.*, 2002; Jobic *et al.*, 2007). Secondly it has been noted in studies using [1-^{13}C]-glucose as a growth substrate, that there is a "scrambling" of the label originating from the C1, which results in label appearing on the C1 and C6 of molecules such as glucose and trehalose (Martin *et al.*, 1985; Martin *et al.*, 1988; Ramstedt *et al.*, 1989; Peksel *et al.*, 2002; Rangel-Castro *et al.*, 2002). This has been explained as occurring via the metabolism of the labelled substrate via mannitol, with the operation of the purported mannitol cycle, and the symmetry of the compound, resulting in the observed scrambling (Martin *et al.*, 1985; Martin *et al.*, 1988; Ramstedt *et al.*, 1989; Rangel-Castro *et al.*, 2002). Given the evidence against the mannitol cycle in *Stagonospora nodorum*, the mannitol mutants created presented an opportunity to re-examine the

metabolic fate of ^{13}C-labelled carbon, and to further elucidate the mechanism of mannitol metabolism.

5.1.4 Aims of the Study

The aims of this study were to characterise the differences between the *S. nodorum* wild type strain SN15, and mutants with a disrupted mannitol dehydrogenase gene (*mdh1-71*), a disrupted mannitol 1-phosphate dehydrogenase gene (*mpd1-1*), or with both of these genes disrupted (*mpd1mdh1-107*) using ^{13}C-NMR spectroscopy. It was hypothesised that differences between the natural abundance spectra, and spectra acquired after growth on [1-^{13}C]-labelled substrates would reveal differences in the pathway(s) of carbon metabolism between the strains which would firstly elucidate how the *mdh1-71* strain is able to catabolise mannitol.

It was further hypothesised that the scrambling of the ^{13}C label seen in other studies, resulting in labelling of both terminal carbons of glucose and trehalose, can occur by either of the mannitol metabolic pathways. In the double mutant, however, this scrambling mechanism will be inoperable, and the presence or absence of label in the C6 of trehalose and glucose will indicate whether other pathways such as the aldose/triosephosphate isomerase/pentose phosphate pathway contribute significantly to scrambling.

5.2 MATERIALS AND METHODS

5.2.1 Preparation of Standards

Compound standards were prepared by making a 60-250 mM solution of the compound in 1 mL D_2O (99 atom %, Sigma-Aldrich Co., St. Louis, MO, USA). The solution was lyophilised overnight in a Savant FDC206 freeze-drying chamber (Savant Scientific Instruments, Farmingdale, NY) attached to an Heto Maxi-Dry Lyo freeze dryer (Heto-Holten, Allerød, Denmark) and stored at -80 °C until required. Prior to NMR spectroscopy the freeze-dried standard was resuspended in 1 mL D_2O, centrifuged at 20800 g for 10 min in a benchtop Eppendorf Centrifuge (Model 5417C, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) to pellet any debris, and 700 μL transferred to a NMR tube for spectroscopy.

5.2.2 Flask Culture of Fungal Strains

The strains SN15, *mdh1-71*, *mpd1-1* and *mpd1mdh1-107* were used in this experiment.

5.2.2.1 Natural Abundance Cultures

Natural abundance (NA) cultures were inoculated with mycelium and spores scraped from 1/3 of a CZV8CS agar plate culture into a 250 mL flask containing 50

mL MM-C liquid medium with 40 mM glucose. An additional set of *mpd1mdh1-107* NA cultures were started with inoculum from minimal medium agar plates. The flasks were incubated on a Certomat® R shaker (B. Braun, Melsungen, W. Germ.) operating at 140 rpm, for 3 days at 20 °C in the dark. Mycelium was harvested after 3 days into a pre-weighed sterile 50 mL Falcon tube and centrifuged for 10 min at 3000 g at 4 °C. The supernatant was discarded and the pellet resuspended in 50 mL milliQ H_2O and centrifuged for a further 10 min at 3000 g at 4 °C. The supernatant was discarded and the pelleted mycelium was snap frozen by placing the tubes in liquid nitrogen. Samples were lyophilised overnight in a Savant FDC206 freeze-drying chamber (Savant Scientific Instruments, Farmingdale, NY) attached to an Heto Maxi-Dry Lyo freeze dryer (Heto-Holten, Allerød, Denmark). Tubes were re-weighed to determine the dry weight of the mycelium, prior to being replaced in liquid nitrogen and stored at -80 °C. Three replicates of each treatment were prepared.

5.2.2.2 [1-^{13}C]-Glucose-Labelled Cultures

Cultures were prepared as for NA cultures (5.2.2.1) except that the carbon source added to the MM-C liquid medium was 40 mM D-[1-^{13}C]-glucose (Sigma-Aldrich Inc., St. Louis, MO).

5.2.2.3 [1-^{13}C]-Mannitol-Labelled Cultures

5.2.2.3.1 Assay of mannitol uptake

Three 50 mL minimal media flask cultures were prepared for each of the strains SN15, *mdh1-71*, *mpd1-1* and *mpd1mdh1-107* and incubated for four days. Cultures were harvested and centrifuged as described above and 1.5 mL of the minimal medium retained for assay from each culture. The cultures were then washed in 50 mM Tris-HCl pH 7.5 and transferred to fresh flasks containing MM-C with 40 mM mannitol. For control purposes a set of flasks were prepared containing MM-C or MM-C with 40 mM mannitol, but to which no fungal culture was added. An aliquot of 1.5 mL was taken from each of these control flasks and retained for assay. All flasks were incubated under standard growth conditions. At 24 h and 48 hours incubation a 1.5 mL aliquot was taken from each flask and retained for assay.

A flask culture of SN15 was grown for three days in 50 mL MM-C + 40 mM sucrose and harvested and prepared for enzyme assay as described above (Section 3.2.4). The mannitol oxidation activity of the mannitol dehydrogenase enzyme (Section 3.2.4.3.3) in the desalted extract was used to determine the amount of mannitol in the spent medium and controls. A standard curve was prepared using 50 µL per 1 mL assay volume of 0, 10, 20, 30, 40 and 50 mM mannitol. The activity of all collected samples was similarly assayed using 50 µL per assay volume. Additional controls using 50 µL milliQ water or 50 µL 20 mM or 40 mM mannitol as the substrate were included in each run. The mannitol standard curve was used to calculate the amount of mannitol in each sample. The allowed the determination of the amount of mannitol uptake for each culture.

5.2.2.3.2 Preparation of [1-^{13}C]-mannitol-labelled cultures

Cultures were prepared as for the natural abundance cultures (5.2.2.1) except that strains were harvested and washed after two days incubation on 40 mM glucose. The harvested mycelium was washed and transferred to a fresh 250 mL flask containing 50 mL MM-C with 20 mM D-[1-^{13}C]-mannitol (Omicron Biochemicals, Inc., South Bend, IN). Cultures were incubated for a further 24 h and then harvested and stored as for natural abundance cultures.

5.2.2.4 [1-^{13}C]-Glucose Feed-Chase Cultures

A set of 12 SN15 flask cultures were incubated for three days, harvested and washed as per the natural abundance cultures (5.2.2.1) The washed cultures were transferred to fresh 250 mL flasks containing 50 mL MM-C with 40 mM D-[1-^{13}C]-glucose (Sigma-Aldrich Inc, St. Louis, MO) and returned to the shaker. At one hour and four hours incubation on the labelled medium, three cultures were harvested, washed and snap frozen in liquid nitrogen for lyophilisation. The six remaining cultures were also harvested and washed after 4 hours on the labelled medium, and then transferred to a further set of 250 mL flasks containing 50 mL MM-C with 40 mM unlabelled glucose. At one hour and four hours post-transfer to the unlabelled medium, three cultures were harvested, washed and snap frozen in liquid nitrogen for lyophilisation. All cultures were lyophilised overnight, weighed, and stored as per the natural abundance cultures.

5.2.3 Methanol/Water Extraction of Polar Metabolites

Samples were retrieved from the -80 ºC freezer and ground in a sterile mortar and pestle with liquid nitrogen to a fine powder. Once ground, 20 mL of a -20 °C 70:30 methanol:water solution was added and the sample was ground for a further 1 minute. The resuspended sample was poured into a funnel lined with #1 Whatman paper filter and the filtrate collected into a flask. The mortar and pestle were rinsed twice with a further 5 mL -20 °C 70:30 methanol:water solution and this was added to the funnel and the filtrate collected. The filtrate volume was reduced by rotary evaporation using an RE111 Rotavapor (Büchi, Switzerland) with the water bath temperature set to 45 °C until about 2-3 mL remained. The samples were transferred to a sterile 10 mL Falcon tube, snap frozen in liquid nitrogen and lyophilised overnight as described above (5.2.2.1) and stored at -80 °C until ready for NMR analysis.

5.2.4 NMR Tube Preparation

Prior to use, NMR tubes (Kontes Glass Company, Vineland, New Jersey, USA) were rinsed 2-3 times with de-ionised water. Tubes were then rinsed 2-3 times with 100% acetone to expel any remaining water and dried overnight in a 150 °C oven.

5.2.5 Sample Preparation for NMR Analysis

Samples were retrieved from the -80 °C freezer, resuspended in 1 mL D_2O (99 atom %, Sigma-Aldrich Inc., St. Louis, MO, USA) and transferred to a pre-weighed 1.5 mL Eppendorf tube. Samples were lyophilised overnight as described previously (5.2.2.1), reweighed to determine the dry weight of the polar extract, and stored at room temperature until ready for NMR analysis. Just prior to NMR spectrometry, samples were resuspended in 1 mL D_2O and centrifuged in a benchtop Eppendorf microcentrifuge at 20800 g for 10 min to pellet any particulate debris. The liquid fraction was transferred to a fresh 1.5 mL Eppendorf tube, re-centrifuged, and 600 µL transferred to a clean NMR tube and taken for NMR spectroscopy. The pellet in the pre-weighed Eppendorf tube was evaporated to dryness in a 68 °C heating block and the tube re-weighed to determine the dry weight of the pellet. This weight was deducted from the original weight of the dried extract to determine the weight of the soluble polar extract in the NMR sample.

5.2.6 NMR Spectra Acquisition

The ^{13}C NMR spectra were acquired using a Bruker Avance DPX-300 Spectrometer (Bruker Instruments Inc, Billerica, MA) operating at 75.5 MHz, equipped with a 5 mm ^{1}H/multinuclear probe, and interfaced with a console running Xwin-NMR Version 3.5. Spectra were routinely run at 300 K with locking on the D_2O solvent. For samples containing carbon standards or ^{13}C-labelled samples, 512 scans were sufficient to obtain a satisfactory spectrum. For natural abundance or very low dry weight samples 10,000 scans were routinely acquired.

5.2.7 NMR Spectra Analysis

5.2.7.1 Software

Spectra were analysed using MestReNova 5.2.1 NMR analysis software (MestreLab Research, A Coruña, Spain). Assignment of spectral peaks to compounds was based upon comparison of their chemical shifts and relative intensities with those of prepared standards or with compounds listed in the Spectral Database for Organic Compounds (SDBS) (AIST, 2008), the Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra (Pouchert and Behnke, 1993) and the Chemical Shift tables compiled by Fan (1996).

5.2.7.2 Compound Identity and Label Quantification

5.2.7.2.1 Internal referencing of chemical shifts

The MestReNova sensitivity threshold for peak selection was adjusted to that level which was sufficiently low to pick up the majority of biologically relevant peaks, but without picking random noise peaks. Negative peaks and peaks with a chemical shift of less than zero were automatically precluded. The chemical shifts of the selected peaks were referenced to the largest peak of the most abundant compound in the spectrum using the values from the compound standard spectra. Typically this was the β-anomer of the carbon 1 (C1) of glucose (95.83 ppm), the C1,6 of mannitol (63.16 ppm) or the C1 of trehalose (93.16 ppm). When possible, the compound which was used as the substrate (glucose or mannitol) was used for this referencing process.

In those instances where the substrate had been exhausted, the principal metabolic product was used (mannitol or trehalose). These were all 6-carbon compounds and the peaks selected had an unambiguous chemical shift, which facilitated their identification.

The re-referenced peak data was downloaded and compared to the library of compound standards. Peaks were initially assigned to compounds where the difference between the chemical shifts of the sample peak and standard peak were less than 0.1 ppm. Peaks which were assigned to the same compound were assessed on the basis of the maximum variation of their relative chemical shifts (MaxVar(RCS)) and the maximum relative peak intensity (Max(RPI)) to confirm their identity.

5.2.7.2.2 MaxVar(RCS)

The MaxVar(RCS) was determined by calculating the differences between the actual chemical shifts for each peak assigned to a compound, and their ideal chemical shifts as established by the compound standard. The smallest difference value was deducted from the largest difference value and where the net difference was less than 0.05 ppm, the peaks were considered to be a good match for the compound. This can be expressed as:

$$MaxVar(RCS) = Max(CS_1\text{-}ICS_1,\ CS_2\text{-}ICS_2, \ldots CS_n\text{-}ICS_n) - Min(CS_1\text{-}ICS_1,\ CS_2\text{-}ICS_2, \ldots CS_n\text{-}ICS_n)$$

Where MaxVar(RCS) = maximum variation in relative chemical shift

Max = maximum of calculated differences

Min = minimum of calculated differences

$CS_{1\text{-}n}$ = chemical shift for carbons 1-n of candidate peaks for a compound

$ICS_{1\text{-}n}$ = ideal chemical shift for carbons 1-n from compound standard

5.2.7.2.3 Max(RPI)

A set of ideal NA relative peak intensities (RPI) for each compound was calculated based on the compound standard NA spectra, assigning the most intense peak (the base peak) a value of 100%. The RPI for all other resonances in the compound standard were determined in relation to the base peak. The RPI for the peaks assigned to a compound in a biological spectrum were determined by applying the intensity of the compound standard base peak (i.e. 100%) to the peak which best matched the base peak in terms of chemical shift. This was not necessarily the most intense peak in the biological candidate peaks. The RPI were calculated for all other peaks assigned to the compound in the biological spectrum. The Max(RPI) was determined by dividing the calculated RPI for each candidate peak by its ideal RPI and deducting 1. Given that there was an observed variation between standards from different sources as noted below (Section 5.3.1), and given further that two peaks from the same compound could have their intensity altered in opposite directions, there was some flexibility required in the use of this parameter. Generally, as long as the RPI of particular candidate peak was within the range of -50% to +80% of the standard, it was not rejected as belonging to that compound. The Max(RPI) can be expressed as:

$Max(RPI) = Max((ORPI_1/IRPI_1)-1), (ORPI_2/IRPI_2)-1),\ldots(ORPI_n/IRPI_n)-1))$

Where Max(RPI) = maximum difference in observed versus ideal relative peak intensities

$ORPI_{1\ldots n}$ = observed relative peak intensity for candidate peaks 1…n

$IRPI_{1\ldots n}$ = ideal relative peak intensity for peaks 1…n of a compound

If a peak was rejected as belonging to the assigned compound, the remaining peaks were reassessed using Max(RPI) to determine whether they constituted acceptable candidates for the compound.

5.2.7.2.4 Missing peaks

Where some peaks were identified for a compound, but others were not present, an iterative search process was undertaken. Where the missing peak(s) were of low intensity, the sensitivity threshold could be lowered to pick up the missing peak(s). If this resulted in too much noise being picked up then the peak was discounted. Where the chemical shift of a candidate peak resulted in MaxVar(RCS) being greater than 0.05 ppm, but less than 0.15 ppm, it was only considered if the peak could be assigned unambiguously, if there was evidence from other biological spectra that this peak was subject to variation in its chemical shift, and if the Max(RPI) did not result in its rejection.

5.2.7.2.5 Comparison of relative abundances between spectra

Direct comparison of the relative abundance of compounds between spectra on the basis of resonance peak intensity was not possible since the scale used is an arbitrary one. To enable quantitative comparisons, the sum of the intensities for all peaks above the detection threshold was calculated. For a compound in the spectrum, the ratio of the sum of the intensities of peaks assigned to the compound to the total intensity for the spectra, enabled the percentage of total intensity to be determined. This can be expressed as:

$$\%TI = (\Sigma(I_1, I_2, \ldots I_n))/(\Sigma I)$$

Where %TI = percentage of total intensity

$I_{1\ldots n}$ = intensity of peak 1…n of a compound

ΣI = sum of all resonance peak intensities above the detection threshold

Unidentified peaks which comprised a high percentage of total intensity were the subject of further analysis to provide information concerning their structure and/or identity. This included visual examination of spectra, and comparison of relative chemical shifts and relative peak intensities between spectra, to determine unidentified peaks which might be associated with each other, and which accounted for consistent proportions of total intensity.

5.2.7.2.6 Quantification of ^{13}C-labelling

Peaks assigned to the same compound were compared to determine whether ^{13}C-labelling, above NA levels, had occurred as a result of growth on ^{13}C-labelled substrate. The RPI of the peaks were calculated using the same formula as for determining Max(RPI) above (Section 5.2.7.2.3). A peak was not considered to be labelled unless it had a Max(RPI) of 100% or more i.e. one-fold labelling or more.

There were circumstances where this approach required modification. These occurred where the base peak in the compound standard was the putatively labelled peak in the biological spectrum, where the base peak was co-located with a peak from another compound preventing unambiguous assignment, or where it was subject to peak splitting under the influence of a heavily labelled neighbouring carbon. In these cases the RPI of all sibling peaks were adjusted using the next most intense peak from the compound standard. This procedure was compromised where there were no sibling peaks to form the basis of the comparison, or where the putatively labelled peak had the same chemical shift as the peak of another compound.

5.3 RESULTS

5.3.1 Standards

Natural abundance ^{13}C-NMR spectra were acquired for a number of compound standards as listed (Table 5.1). While the general position of the resonance peaks

Table 5.1: Standard compounds for which ^{13}C natural abundance NMR spectra were acquired.

Compound	Supplier
250 mM L-alanine	Sigma-Aldrich Inc., St. Louis, MO, USA
250 mM L-arabinose	Sigma-Aldrich Inc., St. Louis, MO, USA
250 mM D-arabitol	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-arginine	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-asparagine	Sigma-Aldrich Inc., St. Louis, MO, USA
37.6 mM L-aspartate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM citric acid	Merck Pty. Ltd., Kilsyth, Vic.
250 mM D-fructose	BDH Laboratory Supplies, Poole, UK
100 mM D-fructose 6-phosphate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM D-galactose	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM D-gluconate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM D-glucose	Merck Pty. Ltd., Kilsyth, Vic.
200 mM D-[1-^{13}C]-glucose	Sigma-Aldrich Inc., St. Louis, MO, USA
75 mM D-glucose 6-phosphate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-glutamate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-glutamine	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM glycerol	Sigma-Aldrich Inc., St. Louis, MO, USA
60 mM inosine	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-malate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM D-mannitol	BDH Laboratory Supplies, Poole, UK
200 mM D-[1-^{13}C]-mannitol	Omicron Biochemicals, Inc., South Bend, IN
100 mM D-mannitol 1-phosphate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM D-mannose	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM meso-erythritol	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-methionine	VWR International Ltd, Poole, UK
200 mM L-ornithine	VWR International Ltd, Poole, UK
200 mM L-phenylalanine	VWR International Ltd, Poole, UK
200 mM pyruvate	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-serine	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM D-sorbitol	VWR International Ltd, Poole, UK
200 mM D-sucrose	Univar, Seven Hills, NSW
200 mM L-threonine	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM D-trehalose	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM L-tryptophan	Sigma-Aldrich Inc., St. Louis, MO, USA
200 mM xylitol	The Sweet Life, Perth, WA

correlated with those reported in the SDBS database, the Aldrich Library, and Fan's Chemical Shift tables, the actual chemical shifts of the peaks were not identical. For example, the C1,6 peak of mannitol was reported as being located at 64.36 ppm (SDBS), 65.99 ppm (Aldrich Library), 73.60 ppm (Fan) and 63.16 ppm (this study). Fan's Chemical Shift tables were compiled from a wide variety of sources and were generally found to agree poorly with the other two resources and from the data collected in this study. Apart from Fan's Chemical Shift tables, the distances between the peaks of mannitol for other spectra, including those quoted in some other studies (Table 5.2), were largely in agreement, with the largest variation being 0.3 ppm.

It was also observed that the relative heights of the peaks exhibited some variation between the spectra of different sources (Figure 5.1). To account for the contribution of peak width to intensity, the relative peak intensities for each compound standard in this study were calculated, assigning the most intense peak a value of 100%. The ACNFP library of compound standards is included in the appendix (Table 8.3). There was still some disagreement between published data in terms of relative peak intensities. For example, while this study and the SDBS agreed that the C2,5 of mannitol was the most intense peak of the three, they disagreed on the order of intensity for the other two peaks of this compound.

There were some instances where the chemical shifts of resonance peaks in different compounds were co-located. This is illustrated by the C5 of L-arabitol and one of the spinning sidebands of [1-^{13}C]-D-mannitol (Figure 5.2).

Table 5.2: ^{13}C-NMR chemical shifts (ppm) for the peaks of D-mannitol from Standard Compound compilations and from reported experimental observations. The calculated differences in the relative positions of the C2,5 and C3,4 – and C2,5 and C1,6, and the net difference in published chemical shifts for each peak, are shown

Data Source	C2,5	C3,4	C2,5 – C3,4	C1,6	C2,5 – C1,6
700 mM Mannitol in D_2O - SDBS (AIST, 2008)	72.05	70.48	1.57	64.36	7.69
Mannitol in D_2O (concentration not given) - Aldrich Library (Pouchert and Behnke, 1993)	73.57	72.00	1.57	65.99	7.58
Mannitol (no concentration or solvent given) (Fan, 1996)	76.3	75.3	1.0	73.6	2.7
200 mM Mannitol in D_2O (this study)	70.74	69.18	1.56	63.16	7.58
Agaricus bisporus (Donker and Braaksma, 1997)	71.7	70.1	1.6	63.9	7.8
Aspergillus nidulans (Dijkema *et al*., 1985)	71.8	70.2	1.6	64.4	7.4
Sphaerosporella brunnea (Martin *et al*., 1988)	72.2	70.6	1.6	64.6	7.6
Magnaporthe [syn. *Pyricularia*] *oryzae* (Yoshida *et al*., 1984)	72.0	70.7	1.3	64.3	7.7
Stagonospora nodorum (this study)*	70.82 (±0.05)	69.25 (±0.05)	1.57	63.22 (±0.05)	7.6
Maximum chemical shift – minimum chemical shift	5.56	6.12	-	10.44	-

* mean (±SE) prior to re-referencing to the internal standard. N ≥ 55.

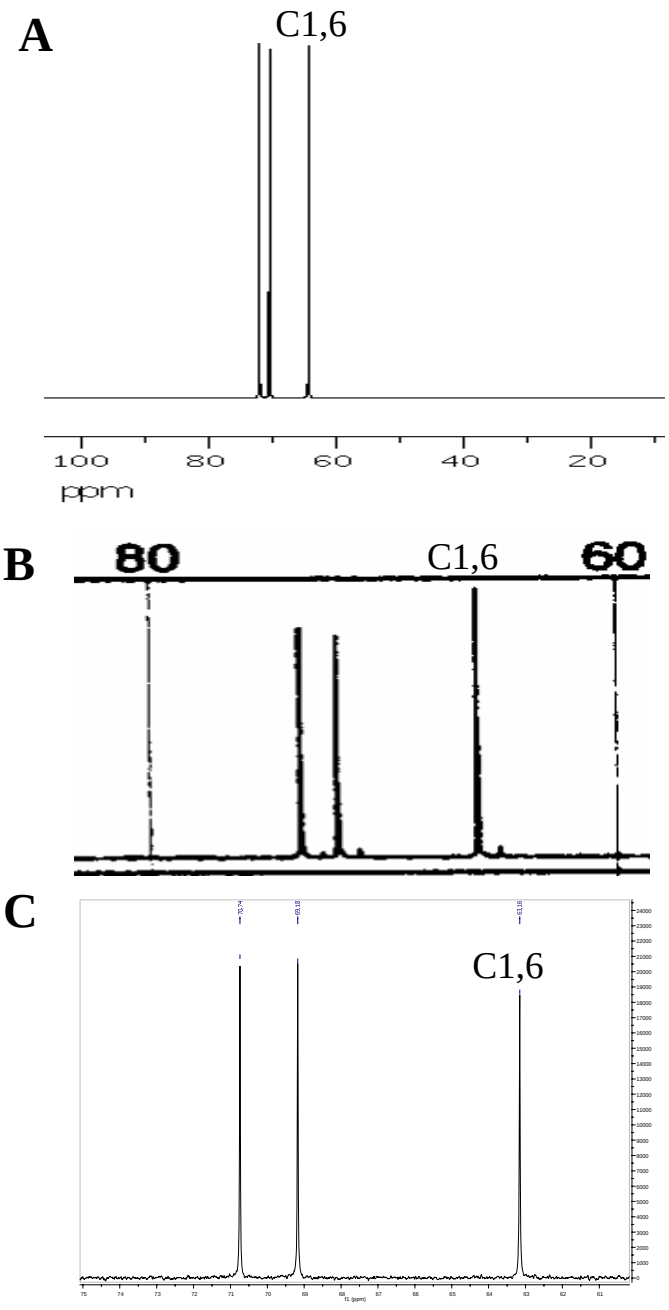


Figure 5.1: ^{13}C NMR spectra for D-mannitol illustrating source-dependent differences in relative height of peaks.

A: Spectral Database for Organic Compounds (AIST, 2008)

B: Aldrich Library of ^{13}C and ^{1}H FT NMR Spectra (Pouchert and Behnke, 1993).

C: This study.

Note the relative height of the C1,6 peak which is the least intense of the three peaks in this study, the most intense in the Aldrich spectrum and of median intensity in the SDBS spectrum.

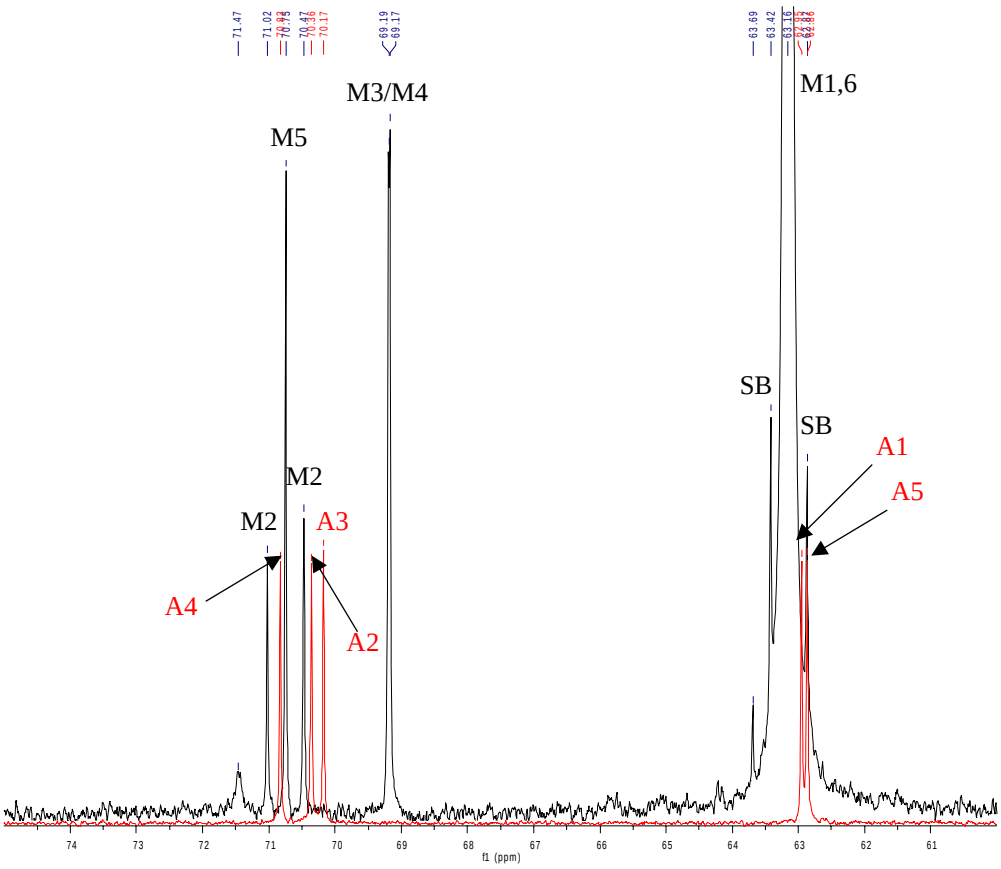


Figure 5.2: ^{13}C-NMR spectra showing co-location of the chemical shifts of the C1 resonance peak of L-arabitol (red) and a spinning sideband of the C1,6 resonance peak of [1-13C]-D-mannitol (black). Abbreviations: A=arabitol; M=mannitol. The M1,6 peak is truncated in this figure. Note that the M2 of mannitol is split and the M3 and M4 peaks have resolved separately under the influence of the 100% labelled M1. The vertical scales have been adjusted to illustrate the situation where a large accumulation of labelled mannitol can obscure the A1 and even the A5 when arabitol is present at a lower abundance.

5.3.2 Identified Compounds

5.3.2.1 ^{13}C Natural Abundance Spectra

5.3.2.1.1 Replicates Inoculated from CZV8CS Agar Cultures

Representative spectra of SN15 and *mpd1mdh1-107* are shown (Figures 5.3 and 5.4). An average of more than 80% of the total intensity for all NA spectra from cultures started with inoculum from CZV8CS plates, was accounted for by nine metabolites. The most abundant identified compounds in the strains, apart from the growth substrate glucose, were glycerol, mannitol or trehalose (Figure 5.5A). Mannitol was the principal metabolite found in SN15 and *mdh1-71* with only traces of glycerol and trehalose detected in these strains. In *mpd1-1* and *mpd1mdh1-107*, the principal metabolite was trehalose, followed by glycerol, and with about 10% of the mannitol content of the wild type. These differences in the major metabolites were statistically significant, with SN15 and *mdh1-71* comprising one group, and *mpd1-1* and *mpd1mdh1-107* forming a second group.

Glucose was detected in all samples (Figure 5.5A) except for one SN15 replicate, and also accounted for less than 10% of total intensity in a second SN15 replicate and one of the *mdh1-71* replicates. In all other samples it accounted for greater than 10% of total intensity. The mean proportions of total intensity were not found to be statistically different between the strains.

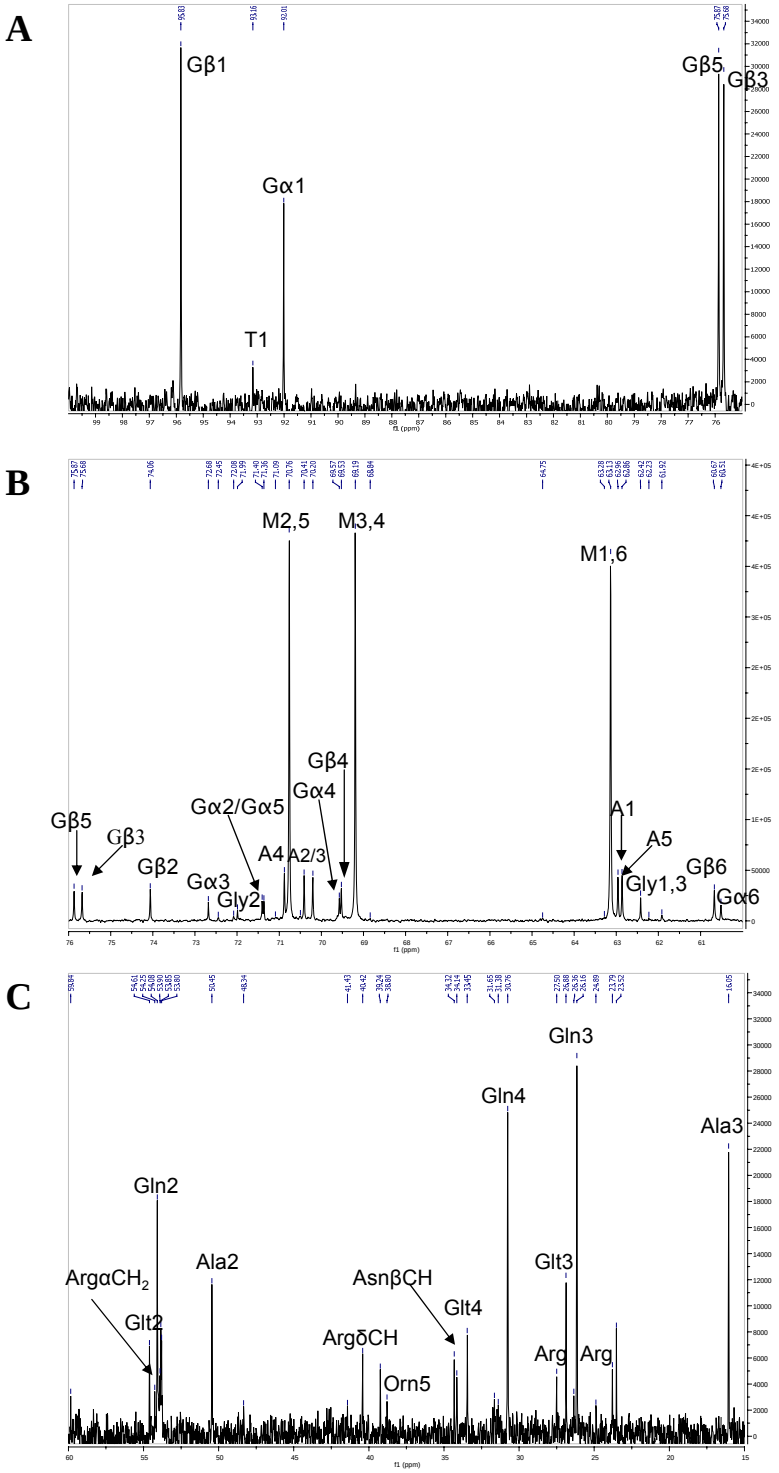


Figure 5.3: Natural abundance ^{13}C NMR spectrum of SN15 showing the regions from **(A)** 75-100 ppm, **(B)** 60-76 ppm and **(C)** 15-60 ppm. Carbons have been assigned using the compound abbreviations A=arabitol; Ala=alanine; Arg= arginine; Asn=asparagine; G=glucose; Gln=glutamine; Glt=glutamate; Gly=glycerol; M=mannitol; Orn=ornithine; T=trehalose. All chemical shifts were referenced to the βC1 peak of glucose. The scales have been adjusted to the height of the most intense peak in each section.

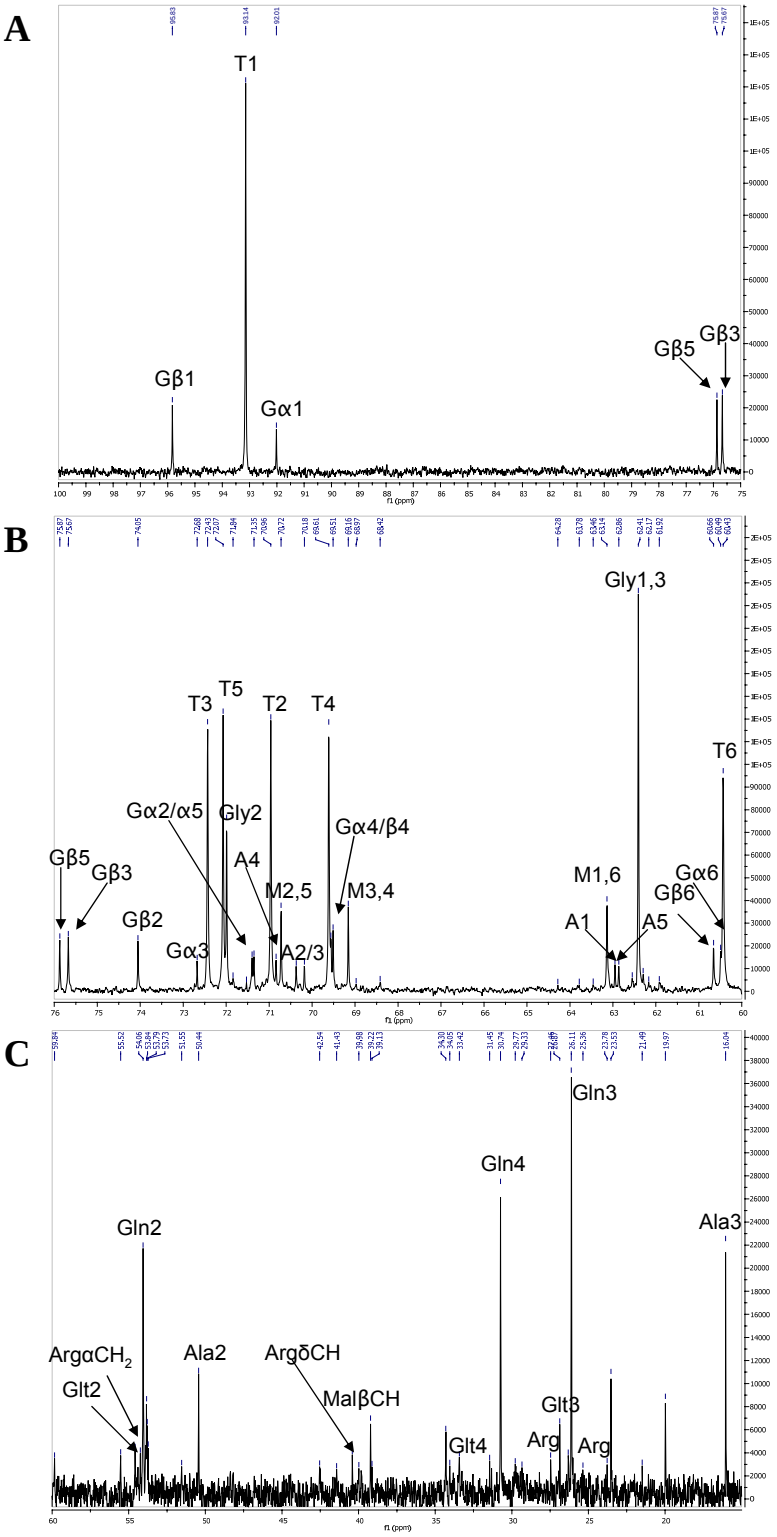


Figure 5.4: Natural abundance ^{13}C NMR spectrum of *mpd1mdh1-107* showing the regions from **(A)** 75-100 ppm, **(B)** 60-76 ppm and **(C)** 15-60 ppm. Carbons have been assigned using the compound abbreviations Ala=alanine; A=arabitol; Arg= arginine; G=glucose; Gln=glutamine; Glt=glutamate; Gly=glycerol; M=mannitol; Mal=malate; T=trehalose. All chemical shifts were referenced to the βC1 peak of glucose. The scales have been adjusted to the height of the most intense peak in each section.

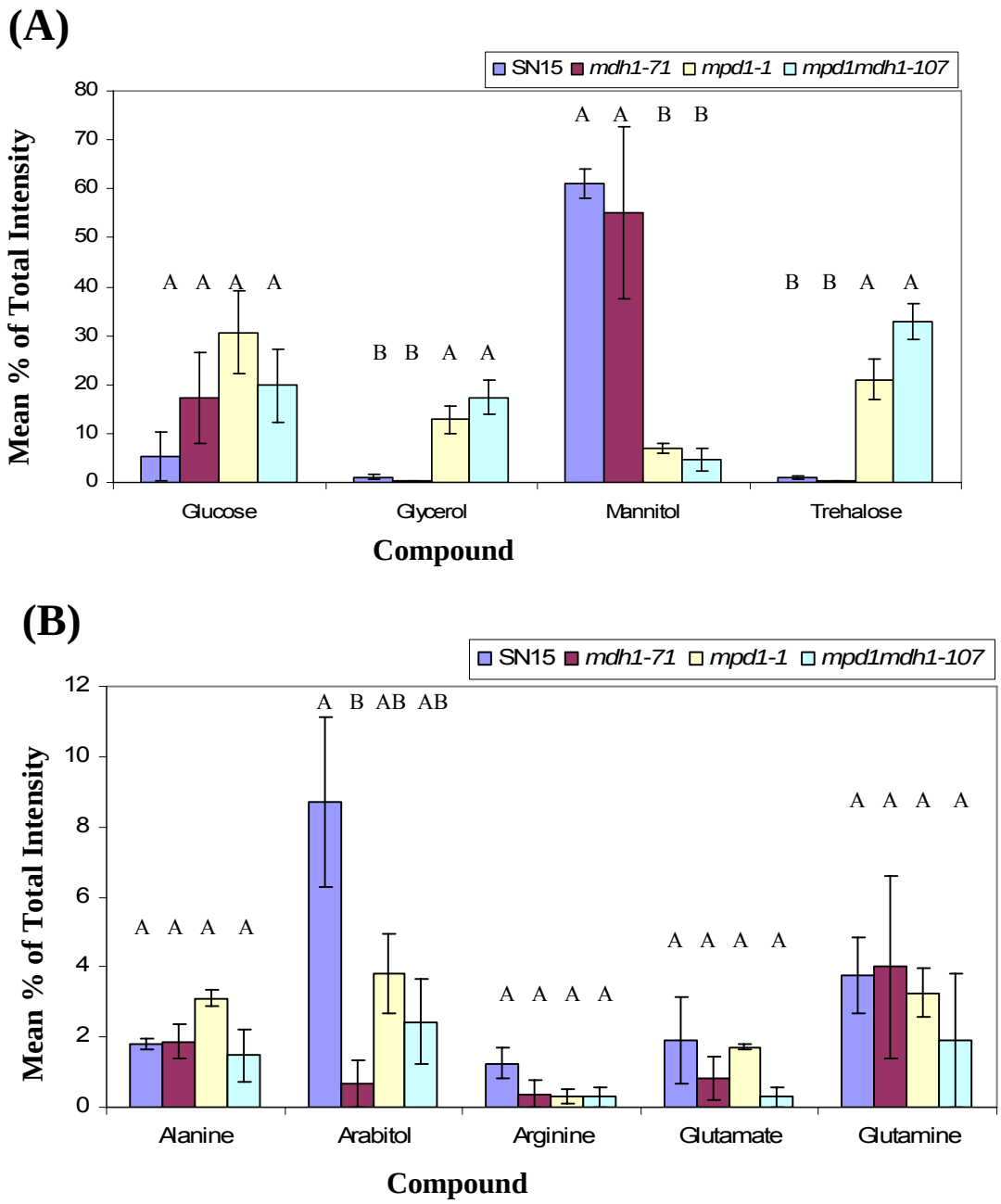


Figure 5.5: Mean relative abundance (±SE) of **(A)** major (>10%), and **(B)** minor (<10%) soluble metabolites in extracts of strains of *Stagonospora nodorum* cultured for 3 days in flasks with 40 mM glucose, as determined by ^{13}C NMR analysis. Relative abundance was expressed in terms of the percentage of total ^{13}C intensity for a spectrum above the sensitivity threshold, which accrued to the compound of interest. Statistical significance was calculated for each group using the Tukey-Kramer HSD test, groups sharing a common letter were not significantly different. N=3.

The five remaining identified metabolites, alanine, arabitol, arginine, glutamate and glutamine, accounted for less than 10% each of total intensity (Figure 5.5B). The relative abundance of each was not significantly different between the strains with the exception of arabitol, which was significantly more abundant in SN15 than in *mdh1-71*.

5.3.2.1.2 Replicates Inoculated from Minimal Medium Agar Cultures

The *mpd1mdh1-107* cultures inoculated from minimal media agar plates exhibited poor growth and their NA ^{13}C-NMR spectra displayed a simpler metabolic profile (Figure 5.6). The total intensity of one replicate was accounted for by glucose (68.6%), glycerol (15.9%), alanine (6.1%), glutamate (1.6%) and unidentified peaks (9.4%), while the second consisted of glucose (64.8%), glycerol (11.6%), alanine (4.1%) glutamate (1.5%) and mannitol (1.1%) and unidentified peaks (16.9%). Apart from glucose, glycerol and alanine, only a single peak was seen for the other assigned compounds. All of the unidentified peaks were situated in the organic acids region of the spectrum or in the aromatic/alkene region, and each accounted for less than 2% of the total intensity.

5.3.2.2 [1-^{13}C]-Glucose-Labelled Spectra

Based on the gross features of the spectra, the strains divided into two groups. The first consisted of SN15 and *mdh1-71*, in which the most prominent peak was the C1,6 of mannitol. A representative spectrum for SN15 is shown (Figure 5.7A). The second group consisted of *mpd1-1* and *mpd1mdh1-107* in which the dominant peak

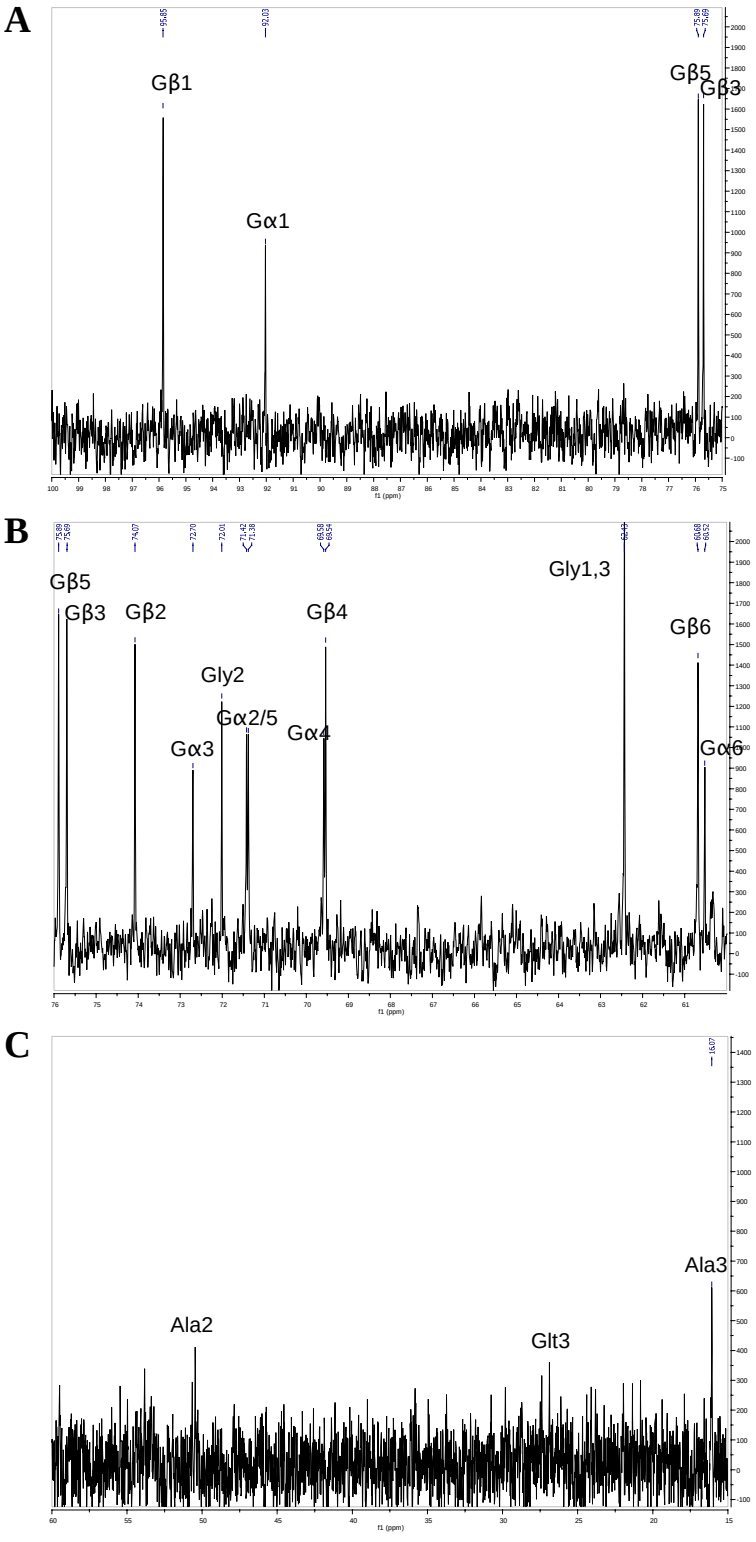


Figure 5.6: Natural abundance ^{13}C NMR spectrum of *mpd1mdh1-107* (inoculum sourced from minimal medium agar plates) showing the regions from **(A)** 75-100 ppm, **(B)** 60-76 ppm and **(C)** 15-60 ppm. Carbons have been assigned using the compound abbreviations Ala=alanine; G=glucose; Glt=glutamate; Gly=glycerol. All chemical shifts were referenced to the βC1 peak of glucose. The scales have been adjusted to the height of the most intense peak in each section.

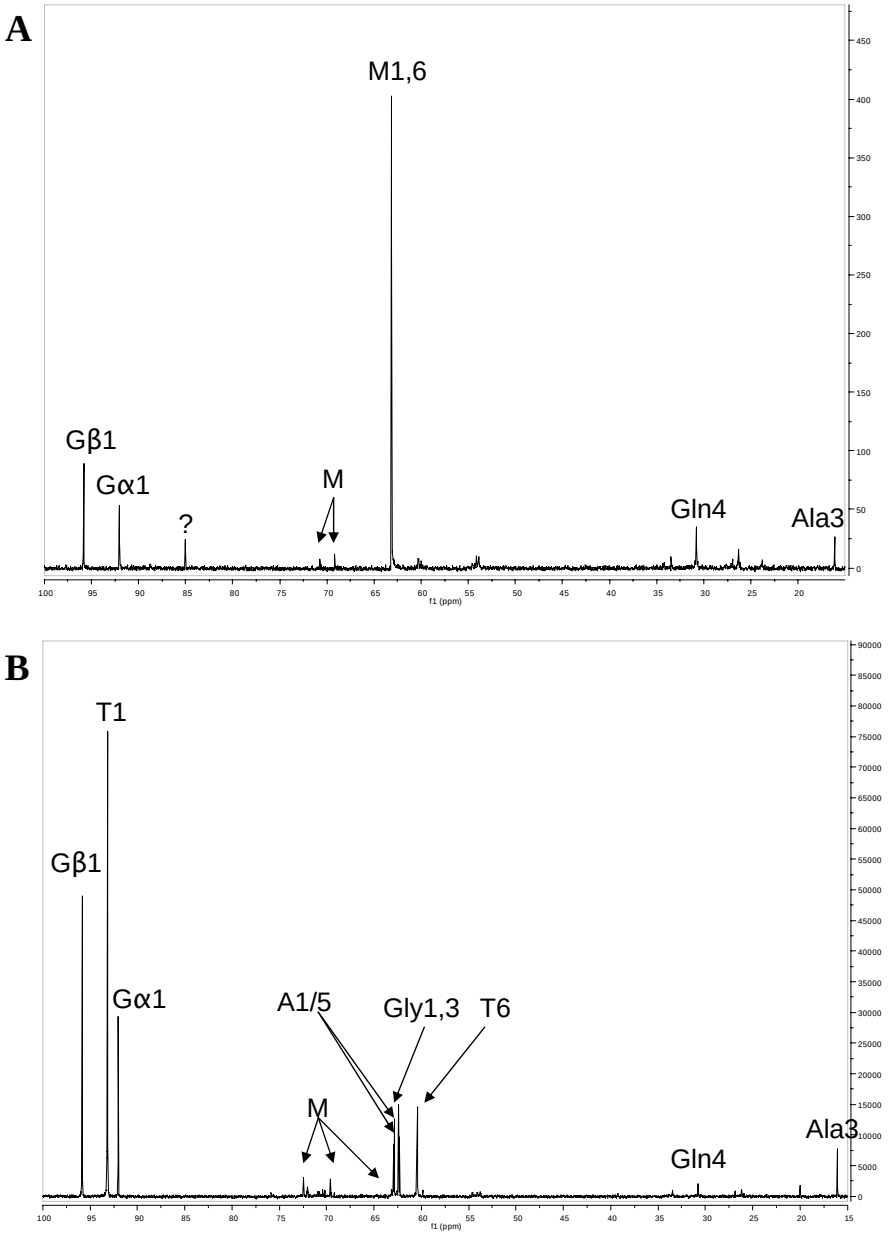


Figure 5.7: ^{13}C-NMR spectra of SN15 (**A**) and *mpd1mdh1-107* (**B**) showing the region from 15-100 ppm for cultures grown for 3 days on [1-^{13}C]-glucose. Carbons have been assigned using the compound abbreviations A=arabitol; Ala=alanine; G=glucose; Gln=glutamine; Gly=glycerol; M=mannitol; T=trehalose; ?=unidentified. All chemical shifts were referenced to the βC1 peak of glucose. The scales have been adjusted to the height of the most intense peak in each section.

was the C1 of trehalose. A representative spectrum for *mpd1mdh1-107* is shown (Figure 5.7B).

5.3.2.2.1 [1-^{13}C]-Glucose

[1-^{13}C]-labelled glucose was present in all samples. In SN15, only the labelled anomeric C1 carbons were identified, and in one sample only a low amount of the β1 anomer was detected. The mean labelling of the C1 carbons in the other strains was determined to be 91.1-fold ± 3.4 above NA (N=9). Spinning sidebands of the C1 carbons were present in most cases, and the β2, α2, β3, β6 and α6 carbon peaks were split.

5.3.2.2.2 Mannitol

All three main mannitol peaks were assigned in all samples. In addition, split C2 peaks were seen in all *mdh1-71* samples as well as the SN15 sample in which almost no glucose was detected. Assuming that labelling of only one terminal carbon contributed to the C1,6 peak, the C1 had a mean fold-labelling above NA of 62.9 ± 13.1 (SN15), 68.6 ± 3.8 (*mdh1-71*), 40.8 ± 5.0 (*mpd1-1*), and was unlabelled in the *mpd1mdh1-107* samples (N≥ 3).

5.3.2.2.3 Trehalose

All trehalose peaks were assigned in all samples of the *mpd1* strains. The C1 had a mean fold-labelling above NA of 32.7 ± 4.8 (*mpd1-1*) and 24.4 ± 1.1

(*mpd1mdh1-107*), while for the C6 this was 6.57 ± 0.9 and 5.02 ± 0.1 respectively (N=3). One *mdh1-71* replicate had a trehalose C1 peak, and one SN15 replicate had C1 and C6 peaks.

5.3.2.2.4 Glycerol

Glycerol was detected in all samples except for one SN15 replicate. For SN15 and *mdh1-71* only the C1,3 peak was assigned, which did not permit the amount of labelling in this peak, if any, to be determined. Both glycerol peaks were assigned in all samples of the other two strains, and the C1,3 peak had a mean fold-labelling above NA of 7.4 ± 0.2 (*mpd1-1*) and 11.0 ± 0.5 (*mpd1mdh1-107*) (N=3).

5.3.2.2.5 Alanine

Alanine was present in all spectra except for the one SN15 sample in which there was almost no glucose detected. In all other cases the C3 peak was present, and for all remaining SN15 samples and one of the *mdh1-71* samples, it was the only alanine peak detected. The intensity of the peak and the absence of the sibling peaks suggested that the C3 peak was labelled. In the remaining samples where sibling peaks were present, it was possible to determine the degree of labelling in the C3 carbon. This was comparable between strains with a mean fold-increase above NA labelling of 13.2 ± 0.8 (*mdh1-71*) (N=2), 16.5 ± 1.3 (*mpd1-1*) (N=3) and 16.0 ± 8.1 (*mpd1mdh1-107*) (N=3).

5.3.2.2.6 Glutamine

This compound was detected in all samples, but the full complement of peaks was found in only one *mdh1-71* spectrum. The C4 of SN15 and *mdh1-71* had a mean of 7.0 ± 1.4 fold labelling above NA (N=7), but this was only 1.0 ± 0.2 for the C4 of *mpd1-1* (N=3), and 1.3 for the only *mpd1mhd1-107* sample for which this could be determined. There was also labelling in the C2 carbon of SN15 and *mdh1-71* of 1.9 ± 0.6 fold above NA (N=7), but no support for labelling of this carbon in the other strains.

5.3.2.2.7 Glutamate

Glutamate was present in all samples apart from the SN15 sample in which almost no glucose was detected. There was no indication of labelling in the compound in any sample.

5.3.2.2.8 Arabitol

The *mpd1mdh1-107* mutant was the only strain in which all carbons of arabitol were detected in all samples. It was calculated that there was labelling in both the C5 (12.7 ± 0.5 fold above NA) and the C1 (8.6 ± 0.3 fold above NA) carbons (N=3). The C5 peak in the other strains was obscured by a mannitol C1,6 sideband, apart from one of the *mpd1-1* samples. With the single exception of one SN15 sample in which all peaks apart from C5 were identified, the only arabitol peak assigned in the samples

of the other strains was the C1. The C1 of the SN15 sample was labelled 1.5-fold above NA.

5.3.2.2.9 Arginine

The full complement of arginine peaks was not present in any sample. No arginine was detected in any *mpd1mdh1-107* sample apart from one with a single C3 peak. There was good support in the *mdh1-71* and *mpd1-1* samples for mean fold-labelling of the C6 peak above NA of 2.3 ± 0.67 (N=3) and 3.7 ± 0.05 (N=2) respectively. The data from SN15 was inconsistent with good match for C6 found in only one replicate, with a calculated 11.8-fold labelling of the peak above NA.

5.3.2.3 [1-^{13}C]-Mannitol-Labelled Spectra

5.3.2.3.1 Assay of mannitol uptake

The ability of strains to utilise [1-^{13}C]-mannitol as a sole carbon substrate was investigated. An assay of mannitol uptake by the strains was undertaken to optimise usage of [1-^{13}C]-mannitol. The mannitol standard curve demonstrated that there was a linear relationship between mannitol concentration and net change in absorbance per min at 340 nm due to mannitol oxidation, over the range of concentrations expected in the medium (Figure 5.8A). The spent glucose media had no more activity than the MM-C sample indicating that no mannitol was secreted by the strains (Figure 5.8B). The 20 mM and 40 mM controls confirmed the accuracy of the assay and the continued activity of the enzyme. After 24 h on 40 mM mannitol, the media aliquots

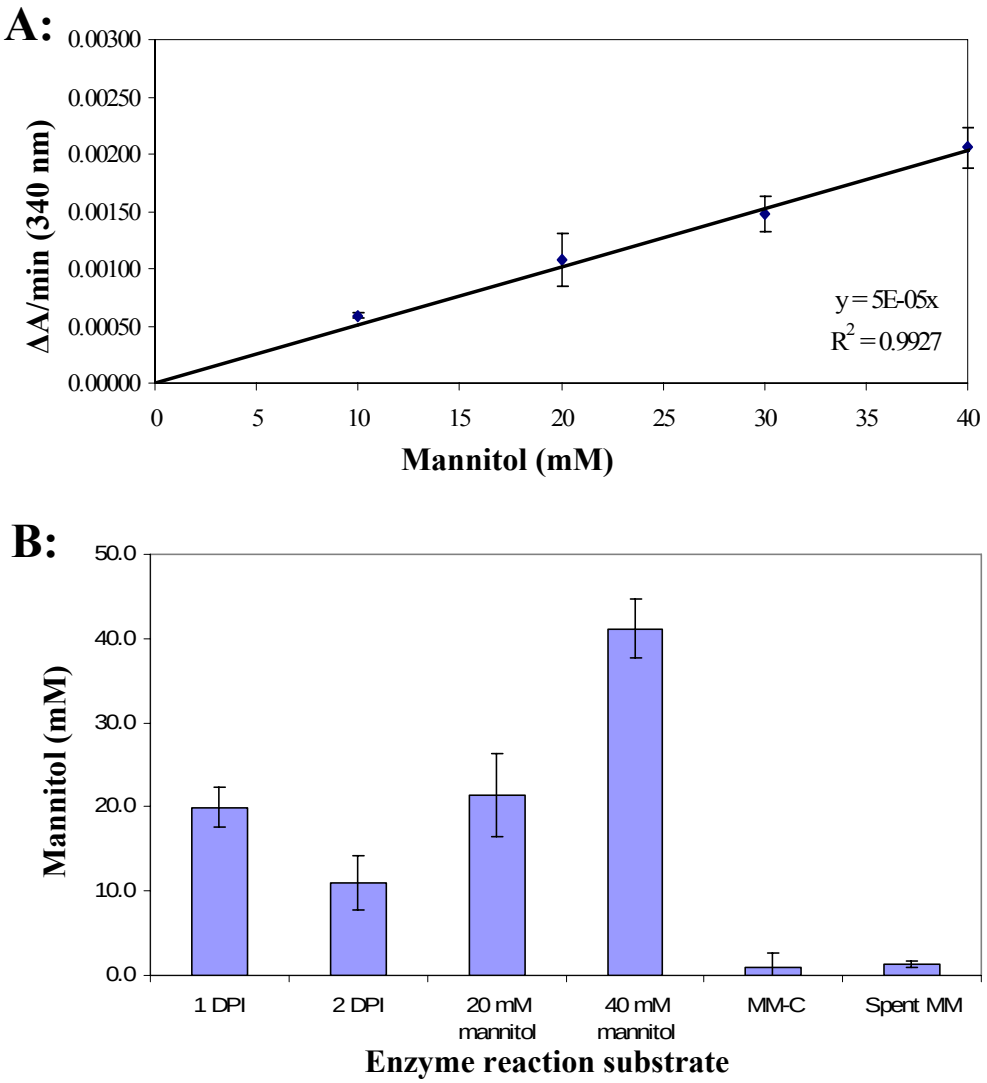


Figure 5.8:

A: Standard curve relating concentration of mannitol to net change in absorbance at 340 nm due to the mannitol oxidation activity of mannitol dehydrogenase in a cell-free extract of *Stagonospora nodorum* strain SN15. N≥ 3.

B: The concentration of mannitol in various samples and controls as determined by observed mannitol oxidation activity in conjunction with the mannitol standard curve above. 1 DPI = mean mannitol concentration in aliquots of minimal media cultures (supplemented with 40 mM mannitol and inoculated with 4 day old flask cultures of strains of *S. nodorum*) taken at 24 hours post-inoculation; 2 DPI = as for 1 DPI but assay performed on aliquots taken at 48 hours post inoculation; 20 mM & 40 mM mannitol = standard controls; MM-C = minimal medium without carbon (medium control); Spent MM = aliquots taken from the 4 day old growth medium from which the fungal cultures were harvested prior to transfer to the mannitol-supplemented media. N≥ 4.

contained a mean concentration of 20.0 ± 8.2 mM mannitol (N=12). This was further reduced after 48 h incubation to a mean concentration of 11.0 ± 9.8 mM mannitol (N=12). As a result, it was determined that 20 mM [1-^{13}C]-mannitol would be sufficient to meet the uptake requirements of all strains for the labelling experiment as planned.

5.3.2.3.2 Gross features of spectra

The gross features of the spectra from the different strains were all similar insofar as the C1,6 peak of mannitol was the most intense resonance peak. A representative spectrum from each strain is shown (Figure 5.9). There were some obvious differences between the strains in that the *mpd1-1* and *mpd1mdh1-107* strains displayed few peaks other than mannitol, while the SN15 and *mdh1-71* spectra had a number of peaks in the sugar/polyol and organic acid regions.

5.3.2.3.3 Mannitol

All peaks of [1-^{13}C]-mannitol were assigned in all samples, including C1,6 sidebands and split C2 peaks, and all with strong labelling above NA of the C1,6 peak. Assuming that only one terminal carbon was labelled, the mean fold-labelling above NA of C1 was 80.0 ± 9.3 (SN15), and 53.84 ± 18.4 (*mdh1-71*) (N=3). The other two strains additionally showed resolved C3 and C4 peaks, otherwise only seen in the compound standard (Figure 5.2), and which were taken to be indicative of 100% labelling of the C1.

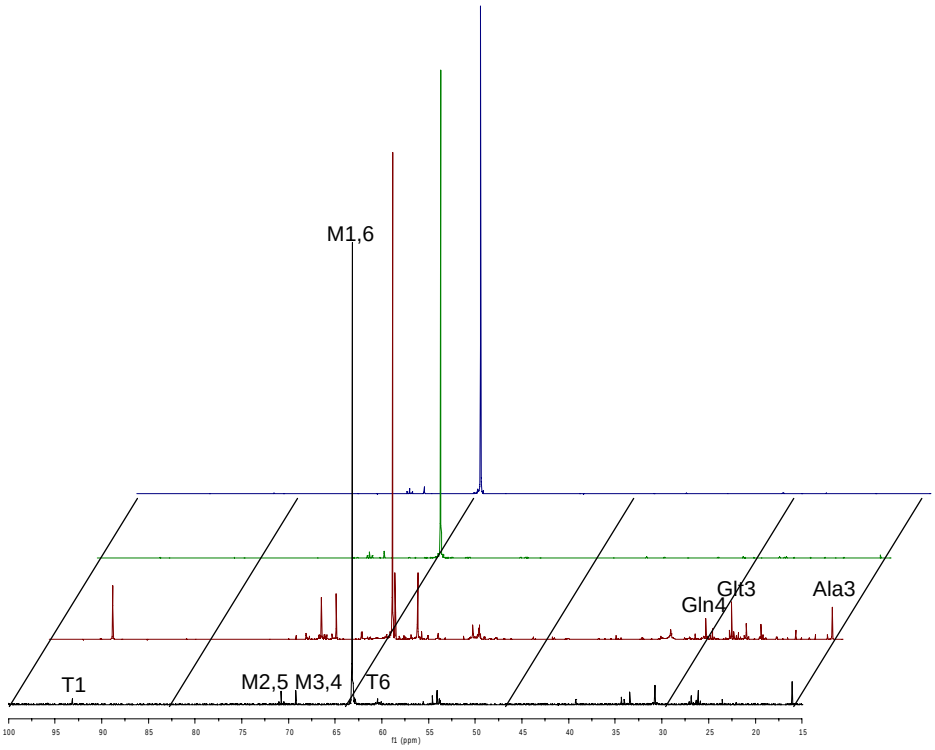


Figure 5.9: ^{13}C-NMR spectra of *Stagonospora nodorum* strains SN15 (black), *mdh1-71* (red), *mpd1-1* (green) and *mpd1mdh1-107* (blue) grown for two days on 40 mM glucose followed by 24 h on 20 mM [1-^{13}C]-mannitol. Each spectrum is representative of three independent experiments. Peak heights have been normalised to the intensities of the C1,6 peaks of mannitol. Assigned peaks in this figure pertain to trehalose (T), mannitol (M), glutamine (Gln), glutamate (Glt) and alanine (Ala) for the carbons as numbered.

5.3.2.3.4 Trehalose

Most trehalose peaks were detected in *mdh1-71* samples, but in most other instances only the C1 and C6 were assigned. In two of the *mpd1mdh1-107* and one of the *mpd1-1* replicates, no trehalose was detected. In the *mdh1-71* replicates, mean fold-labelling of the C1 above NA was 8.0 ± 1.3, and of the C6 was 14.4 ± 5.3 (N=4).

5.3.2.3.5 Glucose

Glucose was only detected in one replicate each of SN15 and *mdh1-71* and in both cases it was only the C1 anomers and βC6 peaks which were seen.

5.3.2.3.6 Glycerol

The C1,3 peak of glycerol was assigned in all *mdh1-71* and two SN15 replicates. No glycerol was detected in any of the *mpd1* mutants.

5.3.2.3.7 Arabitol

The C5 of arabitol was obscured in all spectra. The *mdh1-71* strain was the only one in which arabitol was detected in all replicates. The C1 was not assigned in any SN15 spectrum and labelling of other peaks was not suggested. The C1 of arabitol in the *mdh1-71* spectrum had a mean fold-labelling above NA of 2.6 ± 0.8 (N=4). Labelling of the C1 could only be determined in a single spectrum each of *mpd1-1* and *mpd1mdh1-107* of 5.9-fold and 6.4-fold above NA respectively.

5.3.2.3.8 Amino acids

Alanine was detected in all SN15 and *mdh1-71* spectra. Mean labelling of the C3 in the *mdh1-71*spectra was 17.7 ± 3.6 fold above NA (N=3). It was only possible to determine labelling in one SN15 replicate of 9.9-fold above NA. Alanine was detected in one *mdp1mdh1-107* and two *mpd1-1* spectra. Only the C3 peak was assigned, and its intensity was not suggestive of any labelling.

Most glutamate peaks were seen in all SN15 and *mdh1-71* samples. There was some circumstantial support for two- to three-fold labelling above NA of the C3 in some spectra, but this was contradicted in other replicates. It was only spasmodically detected in the other two strains.

Arginine peaks were seen in spectra of all strains, although they were absent in several spectra. There was no indication of labelling of any peak in spectra where such calculation was possible.

Glutamine was detected in all *mdh1-71* replicates and most SN15 replicates but spasmodic in the other two strains. There was no strong support for labelling above NA in any carbon.

5.3.2.4 [1-^{13}C]-Glucose Feed-Chase Spectra

The SN15 strain was investigated for the *in vitro* metabolic fate of labelled carbon in a feed-chase experiment. The 'feed' phase involved growing cultures on [1-^{13}C]-glucose, with cultures transferred to unlabelled glucose during the 'chase' phase. This technique is used in ^{13}C-NMR to identify the pathways and products of metabolism, and to indicate which metabolite pools are active. Four replicates were prepared for each time-point sampled. However, one of the 1h feed cultures was found to be contaminated at harvest and discarded. A representative spectrum from each time-point is shown (Figure 5.10).

The calculated mean fold-labelling above NA of carbons for which this could be determined is shown (Figure 5.11).

5.3.2.4.1 Carbohydrates

Mannitol was present in all samples. The three main peaks were assigned in all cases, and the split C2 peaks and C1,6 sidebands were assigned in the majority of cases. Assuming that the increased intensity of the C1,6 peak was due to labelling in one terminal carbon only, there was mean labelling above NA after 1 h on labelled glucose substrate of 24.3-fold ± 2.6 (N=3), rising to 38.4-fold ± 2.9 (N=4) after four hours. One hour after transfer to unlabelled glucose this fell to 19.6-fold ± 4.1 (N=4), and 7.0-fold ± 0.1 after four hours (N=4).

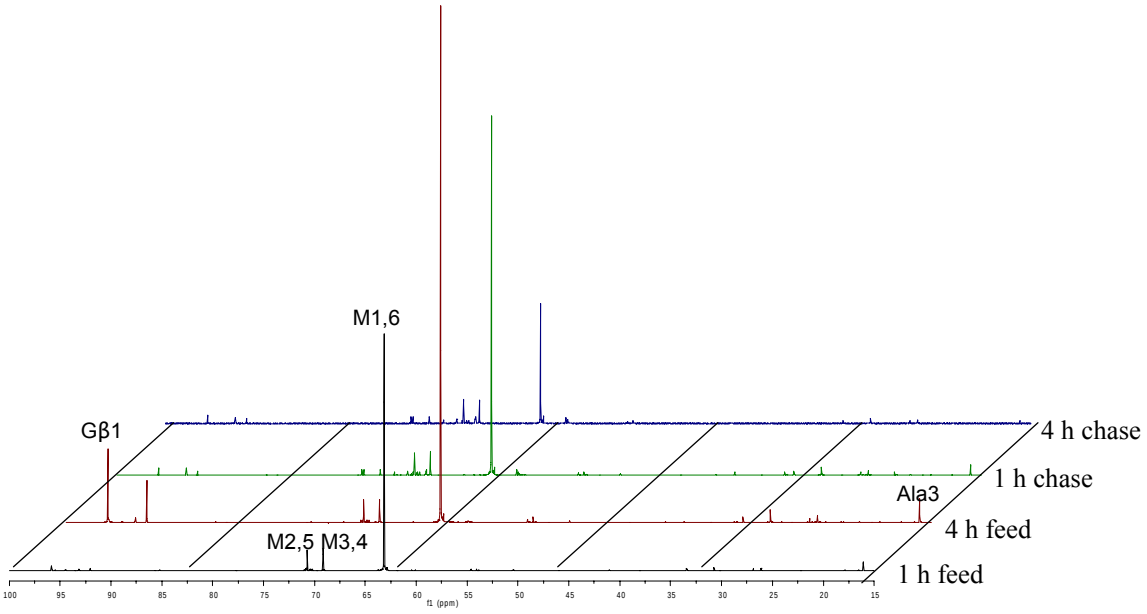


Figure 5.10: ^{13}C-NMR spectra of SN15 cultures from a feed-chase experiment for the range 15-100 ppm. Each spectrum is representative of at least three independent experiments. Peak heights were been normalised to the natural abundance intensities of the C2,5 and C3,4 peaks of mannitol. Cultures represent the ^{13}C label present in spectra after 1 h feed and 4 h feed on 40 mM [1-^{13}C]-glucose and followed by 1h and 4 h chase on 40 mM unlabelled glucose. Assigned peaks in this figure pertain to glucose (G), mannitol (M) and alanine (Ala) for the carbons as numbered.

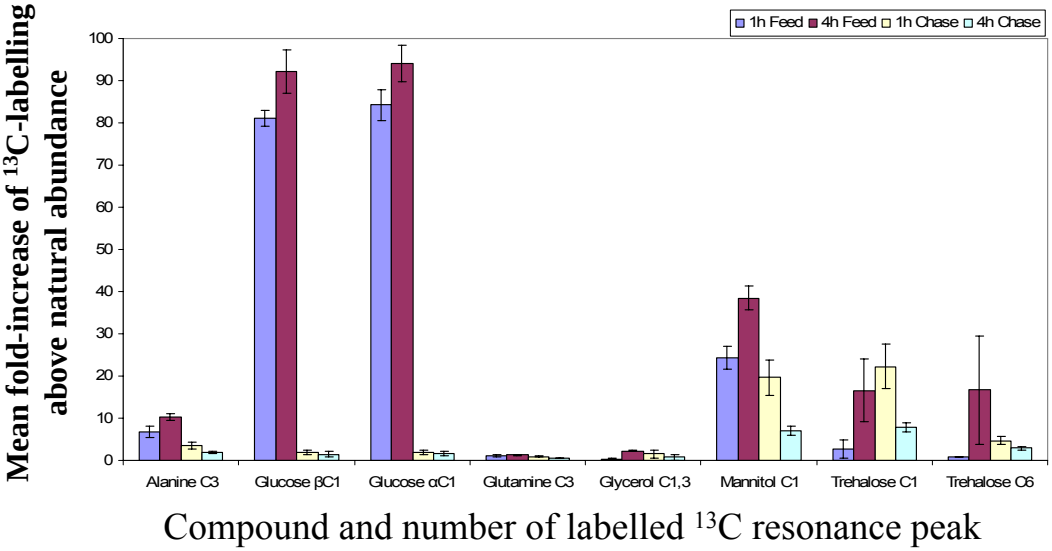


Figure 5.11: Changes in mean (±SE) fold labelling above ^{13}C natural abundance for selected compounds over the course of a feed-chase experiment. Three day old SN15 cultures were transferred to 40 mM [1-13C]-glucose. Half of the cultures were harvested after 1 or 4 hours incubation on the labelled medium and the remainder transferred to unlabelled 40 mM glucose and harvested after 1 or 4 hours incubation on the unlabelled medium. $N \geq 3$.

Glucose was present in all samples. Mean fold-labelling above NA of the βC1 and αC1 carbons was, respectively, 87.4 ± 6.4 and 89.8 ± 5.7 (N=3) after the 1h feed, 92.2 ± 5.1 and 93.4 ± 4.3 (N=4) (4h feed), dropping back to 1.8 ± 0.5 and 1.9 ± 0.6 (N=4) (1h chase) and 1.5 ± 0.6 and 1.6 ± 0.6 (N=4) (4h chase).

Glycerol was detected in all samples, although the lower-intensity C2 carbon was not assigned in one spectrum each from each time period. There was no apparent labelling for the 1h feed or 4h chase samples, but the data supported 2.2 ± 0.1 (N=3)-fold labelling above NA of the C1,3 peak for the 4h feed, and 1.6 ± 1.0 (N=3)-fold labelling for the 1h chase samples.

Trehalose peaks were assigned in all samples, but there was no instance where all six peaks were assigned in a single sample. The C1 peak was the most intense peak in the NA compound standard, and this was the only peak assigned in all samples. In four spectra, the only trehalose peak assigned was the C1 peak. While this is good *prima facie* evidence for its being heavily labelled, it was not possible to calculate its RPI where it was the only peak detected. For the spectra where C1 labelling could be calculated, the pattern was inconsistent. In the 1h feed samples it ranged from 0.5 to 4.8-fold above NA. In the 4h feed samples it ranged from a mean of 4.9-fold (±0.5) in two samples to a mean of 28.2-fold (±7.5) in another two samples. In the 1h chase, there were only two samples where the RPI of C1 could be calculated, and these had a mean fold-increase of 22.3 ± 5.3 above NA. The 4h chase samples were the most consistent with a mean fold-increase of 7.9 ±1.1 above NA for the three samples where this could be determined. There was also the indication of labelling on the C6 of trehalose. This only occurred after the 1h feed and showed great variation after the

4h feed with a mean of 16.6 ±12.9 (N=3). It was more consistent during the chase phase with a mean fold-labelling above NA of 4.7 ±1.0 (N=2) after 1h and 2.9 ±0.4 (N=3) after 4 h.

The C5 of arabitol could not be unambiguously identified as it was co-located with one of the sidebands of the labelled mannitol C1,6 peak. All remaining arabitol peaks were identified in all samples with the exception of the C1, which was not assigned in two of the 1h feed samples, and one of the 4h chase samples. There was no strong evidence for labelling above NA in any of the assigned arabitol peaks.

5.3.2.4.2 Amino acids

All peaks of alanine were assigned in all samples with the exception of the low intensity C1 carbon, which was missing in one replicate each of the 1h feed and 4h feed spectra. The mean fold-labelling above NA in the C3 carbon was 6.8 ± 1.4 after the 1h feed, 10.3 ± 0.9 (4h feed), 3.4 ± 0.8 (1h chase), and 2.0 ± 0.3 (4h chase) ($N \geq 3$).

Glutamine was detected in all samples, although the low-field low-RPI C1 and C5 peaks were not assigned in all cases. The RPI of the C4 carbon was consistently elevated in the feed samples relative to the chase samples and there was support for mean fold-labelling above NA in this carbon of 1.20 ± 0.1 for the feed phase of the experiment (N=7).

The full complement of glutamate peaks was found in all samples and there was no indication of labelling.

The full complement of arginine peaks was not present in any sample, and the compound was not detected in almost half of the samples. In those samples where it was present, there was no indication of labelling.

5.3.3 Miscellaneous Peaks

There were 2,977 peaks across all spectra which were not assigned to any compound with confidence. Of these, 139 represented more than 1% each of the total intensity of the spectrum in which they were found. Forty-two of these peaks could be grouped into 12 clusters of peaks which were within a range of 0.10 ppm (Table 5.3). The majority of these clusters did not have a good match in the Compound Standard Library. The clustering process was extended to include all peaks in the sugar/polyol and organic acid regions of the spectrum (0-100 ppm). This resulted in 1766 peaks being grouped into 146 clusters, each comprising peaks from 3-43 spectra within a range of 0.12 ppm (Table 5.4). Twenty-eight of these clusters had good matches from the Compound Standard Library. However, these were not always unambiguous, and there was insufficient support from sibling peaks to justify identification of a compound in any one spectrum.

Nine of the clusters were comprised of spectra from only one strain and were all located in the organic acids region. Eight of these clusters were specific to SN15.

Table: 5.3: Peak clusters from ^{13}C-NMR spectra of strains of *Stagonospora nodorum* for peaks comprising >1% of total intensity. The range for each cluster, the number of spectra comprising each cluster, the strains and treatments (including the number of replicates), and the best match for the cluster from the ACNFP Compound Standard Library are shown.

Cluster Range (ppm)	No.	Strain/Treatment* + (no. of replicates)	Best Library Match
85.01	6	SN15-^{13}G (5); *mpd1-1*-^{13}G (1)	None
63.29-63.32	4	SN15-^{13}G (1); *mpd1mdh1-107*-^{13}G (3)	Fructose βC1/ Mannitol 1-phosphate
59.83-59.88	4	SN15-^{13}G (3); *mpd1-1*-^{13}G (1)	None
53.90-53.91	3	SN15-^{13}G (1); *mdh1-1-71*-NA (1); *mdh1-1-71*-^{13}M (1)	Methionine αCH
53.84-53.85	6	SN15-^{13}G (2); SN15-^{13}M (1); *mdh1-1-71*-^{13}M (2); *mpd1mdh1-107*-NA (1)	None
53.80	3	SN15-^{13}G (1); SN15-^{13}M (1); *mpd1mdh1-107*-NA (1)	None
53.75	2	SN15-^{13}G (1); SN15-^{13}M (1)	None
29.62-29.63	3	SN15-^{13}M (1); *mdh1-1-71*-^{13}M (2)	Methionine βCH_2
28.86-28.92	2	*mdh1-1-71*-^{13}M (2)	None
26.29	4	SN15-^{13}G (3); SN15-^{13}G (1)	None
25.28-25.29	2	*mdh1-1-71*-^{13}M (2)	None
23.63-23.69	4	*mdh1-1-71*-^{13}M (3); *mpd1mdh1-107*-NA (1)	None

***** NA = natural abundance spectrum from growth on unlabelled glucose; ^{13}G – spectrum from growth on [1-^{13}C]-glucose; ^{13}M – spectrum from growth on [1-^{13}C]-mannitol.

Table 5.4: Distribution of unidentified peaks from ^{13}C-NMR spectra of strains of *Stagonospora nodorum* into clusters.

No. of Spectra per Cluster	No. of Clusters
> 40	4
30-39	6
20-29	10
10-19	59
3-9	67
Total	**146**

Three of the SN15 peaks came from natural abundance spectra while the remainder were from labelled spectra. None of the SN15 clusters had a match in the compound standard library. One cluster was specific to *mdh1-71* and comprised 3 peaks from [1-^{13}C]-mannitol labelled spectra and was a good match for asparagine. No peak accounted for more than 0.3% of total intensity. A further 21 clusters were only found in strains with an intact *Mpd1* gene. Seven of these were located in the sugar/polyol region of the spectrum with one unsupported match for the αC1 of mannose. Of the peaks in the organic acids region, there was one unsupported match for the C2 of threonine, and another for the βCH_3 of pyruvate. There were no clusters which were specific to either or both of the *mpd1* mutants.

There were a number of unidentified peaks which appeared to be labelled based on their intensity. A peak at 85.01 ppm was only noted in spectra of cultures grown on ^{13}C-labelled medium, and accounted for 1.6-6.4% of total intensity in 5 SN15 spectra and 1 *mpd1-1* spectrum. It was present in a further 7 SN15 and 2 *mdh1-71* spectra at lower intensities, and was not observed in any *mpd1mdh1-107* spectrum.

A peak at 62.31 ppm appeared to be labelled in *mpd1mdh1-107* cultures grown on labelled glucose. This peak accounted for 2.4-4.4% of total intensity in all replicates and was present at much lower intensity, or undetectable in natural abundance spectra. It was also seen in several other SN15 and *mdh1-71* spectra, including labelled spectra, but accounted for at least an order of magnitude less of total intensity than was the case for *mpd1mdh1-107*. The best match for this peak was

either the C1 of sorbitol or the αC6 of fructose, but neither match was supported by associated peaks.

There were a number of peaks in the range 58.70-60.80 which accounted greater than 1% of total intensity in spectra of SN15 and *mdh1-71*. They did not justify grouping into clusters, and other strains were represented in the same region at lower intensities. Some presented good matches to the C6 of galactose and mannose or the C2 of threonine, but without good support for this identification.

The two clusters matching methionine from Table 5.3 had additional, lower intensity peaks from all strains. Their identity was not supported by the absence of the sulphonyl carbon in natural abundance spectra, and inconsistent RPI pattern of the two tentatively identified peaks.

The cluster which appeared in most spectra (43) was located at 22.01 ppm and had no match.

5.4 DISCUSSION

5.4.1 Disruption of *Mpd1* Alters the Metabolite Profile

^{13}C natural abundance NMR was used to characterise differences in the metabolite profiles of the *Stagonospora nodorum* wild type strain SN15, and three mutant strains with disrupted mannitol metabolism genes. On average, over 80% of

the observable ^{13}C in the spectra for all cultures started with inoculum from CZV8CS agar plates, was accounted for by nine metabolites. These were divided into two groups comprising the major metabolites (accounting for a mean percentage of total intensity of >10% for the spectra of any strain) and minor metabolites (accounting for a mean percentage of total intensity of no more than 10% for the spectra of any strain).

The NA spectra for the *mpd1mdh1-107* cultures started with inoculum from minimal medium agar plates were designed to eliminate exogenously accumulated mannitol as a factor in these spectra. While this was achieved, the growth was much reduced compared to the same strain on the more complex medium. The presence of glycerol but not trehalose in these spectra may have some implications regarding the relative importance of the role(s) of mannitol for which these compounds appear to be compensating. While this is not uninteresting, these spectra were not strictly comparable with the other NA spectra. The poor growth and largely uninformative metabolite profile of these cultures led to this avenue of investigation being discontinued. All remaining discussion refers to the CZV8CS-derived cultures only.

5.4.1.1 Mannitol

The strains could be divided into two statistically significant groups on the basis of the major metabolites present. Mannitol was the most abundant metabolite in strains with an intact *Mpd1* gene, encoding mannitol 1-phosphate dehydrogenase (SN15 and *mdh1-71*), while this compound was present at an order of magnitude less in the *mpd1* mutant strains (*mpd1-1* and *mpd1mdh1-107*). This is consistent with a

previous ^{13}C-NMR natural abundance (NA) study involving SN15 and *mpd1-1* in *S. nodorum* (Solomon *et al.*, 2005a). It was demonstrated in Chapter 3 that the double mutant strain is unable to synthesise or catabolise mannitol, but can accumulate it from its environment and maintain a stable pool. The detection of low levels of mannitol in all but one of the *mpd1mdh1-107* NA samples implied that this was carried over with the inoculum. The labelling component of this experiment further confirmed this mannitol accumulation behaviour. In the [1-^{13}C]-glucose cultures, the *mpd1mdh1-107* mannitol pool accounted for less than 1% of total intensity in all cases and was unlabelled, while in the [1-^{13}C]-mannitol cultures, this pool accounted for up to 99% of total intensity and was essentially 100% labelled. This accumulation behaviour is not universal for fungi. Germinating spores of the arbuscular mycorrhizal fungus *Glomus intraradices*, did not take up exogenous mannitol under conditions of asymbiotic growth (Bago *et al.*, 1999).

In the case of the *mpd1-1* strain, growth on [1-^{13}C]-glucose indicated that mannitol synthesis was still possible via the mannitol dehydrogenase (Mdh1) pathway, since the C1,6 of mannitol in the strains was labelled 40-fold above NA levels. This confirms the conclusion in Chapter 3 above, that this strain was capable of synthesising mannitol, since it was able to sporulate *in vitro* when serially subcultured onto minimal medium from which exogenous mannitol was absent. The strain was also capable of taking up mannitol from the media as evidenced by growth on [1-^{13}C]-mannitol, with mannitol accounting for up to 99% of total intensity and being essentially 100% labelled.

5.4.1.2 Trehalose and Glycerol

The main metabolites in the *mpd1* mutants were trehalose and glycerol, with both of these being either undetectable, or representing less than 2% of total intensity, in the SN15 and *mdh1-71* spectra. The virtual replacement of mannitol with trehalose and glycerol suggests an alteration in metabolism to compensate for the loss of the ability to accumulate mannitol to wild type levels. Evidently these compounds were unable to repair the inability to sporulate *in vitro* of the double mutant. A number of the postulated roles of these compounds overlap, but not all have been conclusively proven, and they vary in distribution and abundance between species. Targeted gene deletion mutants are elucidating these roles in fungi.

Trehalose 6-phosphate synthase (*Tps1*) has been knocked out in a number of fungi including *S. nodorum*. Some mutants have been more susceptible to environmental stresses including heat (*Botrytis cinerea* and *S. nodorum* (Doehlemann *et al.*, 2006; Lowe, 2006), but not *Magnaporthe grisea* (Foster *et al.*, 2003)), and oxidative stress (*Aspergillus nidulans*, *Candida albicans* and *S. nodorum* (Fillinger *et al.*, 2001; Gonzalez-Parraga *et al.*, 2003; Lowe, 2006; Martinez-Esparza *et al.*, 2007)). Mutants have also been shown to be affected in pathogenicity (*C. albicans*, *Cryptococcus neoformans*, *M. grisea*, and *S. nodorum* (Zaragoza *et al.*, 1998; Foster *et al.*, 2003; Lowe, 2006; Petzold *et al.*, 2006; Wilson *et al.*, 2007), but not *B. cinerea* (Doehlemann *et al.*, 2006)). A more general role for trehalose, ascribed in a wide range of fungal species, is as a storage carbohydrate (Thevelein, 1984; Bécard *et al.*, 1991).

Glycerol is the main compatible solute in the halophilic yeast *Hortaea werneckii* and while erythritol, arabitol and mannitol are all present at optimal growth salinities, only erythritol and glycerol are present during growth on 25% (w/v) NaCl (Kogej *et al.*, 2007). The filamentous fungus *Cladosporium fulvum* accumulated glycerol and arabitol, but not mannitol, in response to osmotic stress (Clark *et al.*, 2003). *Aspergillus nidulans* strains with a disrupted $NADP^+$-dependent glycerol dehydrogenase gene, had strongly decreased levels of glycerol and elevated levels of arabitol, erythritol and mannitol, and exhibited reduced growth on 1 M NaCl (de Vries *et al.*, 2003). Exogenous supplementation with any of these polyols corrected for the deficiency, but uptake of all except glycerol was subject to glucose repression. Osmotic stress resulted in significantly increased arabitol levels in a xylitol dehydrogenase mutant (*xhd1*) of *S. nodorum*, while glycerol was significantly increased in an L-arabitol dehydrogenase mutant (*abd1*), and a double mutant strain (*abd1xdh1*) in which both of these genes had been disrupted (Lowe *et al.*, 2008). Glycerol has also been demonstrated to have roles in fungal phytopathogenicity as the source of the tremendous turgor pressure in appressoria of *M. grisea* (Dixon *et al.*, 1999), and as a nutrient transferred from the host by *Colletotrichum gloeosporioides* f.sp. *malvae* (Wei *et al.*, 2004). Glycerol was shown to accumulate in sunflower cotyledons when infected with *Sclerotinia sclerotiorum*, while uninfected cotyledons and the *in vitro*-cultivated fungus had no detectable glycerol (Jobic *et al.*, 2007).

It might be expected that fungal species in which no Mpd1 activity is present, would show a similar metabolite profile to the *S. nodorum mpd1* mutants. Investigations into the enzymes of mannitol metabolism have demonstrated an absence of Mpd1 activity in the majority of basidiomycetes for which this enzyme

was assayed, although mannitol-1-phosphate phosphatase activity was detected in nearly half of these (Table 1.3). In the case of *Agaricus bisporus*, neither enzyme was detected (Hult *et al.*, 1980) which suggests that only the Mdh1 pathway is active in this species. While only low levels of mannitol were detected in the mycelium of *A. bisporus*, it was the main soluble metabolite in the sporophore and was accumulated as the sporophore developed (Hammond and Nichols, 1976), but decreased post-harvest (Donker and Braaksma, 1997). Trehalose was the second most abundant metabolite, but levels decreased in both mycelium and sporophore as the latter developed (Hammond and Nichols, 1976), and it could not be quantified reliably in ^{13}C-NMR NA spectra post-harvest as most peaks were below the detection threshold (Donker and Braaksma, 1997). Glycerol was not noted as a significant metabolite in these studies. It is therefore apparent that the increase in trehalose and glycerol seen in the *S. nodorum mpd1* mutants is not universally observed in organisms in which only the Mdh1 pathway is active.

The fact that two compounds were accumulated in *S. nodorum* in response to the abolition of mannitol synthesis, implies that there are at least two separate roles of mannitol for which compensation is being made. Further, given that the defect in *in vitro* sporulation of the *mpd1mdh1-107* strain was not repaired by this altered metabolome, there is circumstantial evidence for three roles of mannitol. This could be considered an efficient use of a normally abundant resource. While it may be tempting to consider that glycerol and trehalose are performing the roles of compatible solute and carbohydrate store respectively, this is speculation without specific manipulation of the experimental system to induce conditions which would confirm or refute this. These roles have also not otherwise been conclusively

demonstrated for mannitol. The observation of these differences provides an opportunity to explore the roles of these compounds further. The demonstrated ability of *S. nodorum* gene disruption constructs to abolish either trehalose (Lowe, 2006) or mannitol synthesis (this study), and the implication that trehalose replaces the function of mannitol at least in part, suggests the possibility of combining these constructs in a mutant strain. The production of a triple mutant in this fungus is not a trivial matter, however, and attempts to produce a double mutant harbouring the *mpd1* and *tps1* constructs have thus far proven unsuccessful (Dr. P. Solomon, pers. comm.). While this is suggestive of a lethal condition, this is speculative at this stage.

5.4.1.3 Glucose

This compound occupies an ambiguous position in the NA and [1-^{13}C]-glucose experiments as both the substrate, and as a metabolite. It has previously been detected in ^{13}C natural abundance NMR spectra (Dijkema and Visser, 1987; Solomon *et al.*, 2005a; Jobic *et al.*, 2007), but there is little discussion as to whether it is the result of poorly washed mycelium, substrate which has been taken up but not yet metabolised, the result of *de novo* synthesis via gluconeogenesis, trehalose degradation etc., or some combination of these. It is difficult to justify a claim for any of these scenaria for growth on unlabelled glucose. The fact that one of the SN15 samples had no detectable glucose could be suggestive of variable efficiency in the washing technique. However, this was also the sample with the heaviest extract dry weight of 52.2 mg, more than twice that of all other samples bar one. It is more likely that this sample represented a culture which had exhausted the glucose in the medium. The argument is not trivial, since the degree of labelling of the C1 and C6 of glucose

following growth on [1-^{13}C]-glucose, was used in several studies to estimate the flux of carbon through pathways which cause such scrambling.

The observed pattern of ^{13}C labelling of glucose in the SN15 feed-chase experiment offers some insight into the capacity of mycelium to take up carbon from their environment. An extreme condition must first be considered where the mycelium has virtually no capacity for glucose storage and that [1-^{13}C]-glucose taken up is metabolised immediately. In this instance one would expect to see no labelled glucose in the spectrum of a properly-washed sample, while in an insufficiently washed sample, any glucose present would have 100% labelling of the C1 carbons. However, the mean fold-labelling above NA of the βC1 anomer of glucose in the spectra was 87.4 ± 6.4 after 1h on glucose and 92.2 ± 5.1 after 4 hours (N≥ 3). This is consistent with the addition of [1-^{13}C]-glucose to an initially unlabelled pool of accumulated glucose and with the proportion of labelled substrate increasing with time of exposure to media containing it. Similarly, in samples which were subsequently transferred to medium containing unlabelled glucose, the mean fold-labelling above NA of the βC1 anomer of glucose in the spectra was 1.8 ± 0.5 after 1 h and 1.5 ± 0.6 after 4 h (N=4). This is consistent with the ongoing dilution of label by uptake of unlabelled glucose. If the majority of observed label had been due to poor washing, then it would be expected that the differences in labelling between the 1 h and 4 h samples would be reversed, since the fungus would have reduced the amount of carbon in the medium in that time. The fact that the amount of labelling approached saturation on the labelled medium, followed by its almost complete disappearance on the unlabelled medium, provided evidence that the glucose pool is a transient one with a rapid turnover.

5.4.1.4 Arabitol and Amino Acids

The remaining minor metabolites of the spectra were largely unremarkable in that the amino acids have been identified in ^{13}C-NMR studies in other fungal species (Donker and Braaksma, 1997; Ceccaroli *et al.*, 2003; Jobic *et al.*, 2007), and were not present in significantly different amounts between the different *S. nodorum* strains.

The one exception to this was arabitol, where the NA spectra showed SN15 as having significantly more of this compound than *mdh1-71*. The ability of all strains to produce this compound, however, was confirmed by the labelled spectra in which the C1 peak was routinely observed. The distribution of arabitol as determined by ^{13}C-NMR studies of other filamentous fungal species indicated that this polyol was often absent or below the threshold of detection (Martin *et al.*, 1984; Yoshida *et al.*, 1984; Martin *et al.*, 1985; Martin *et al.*, 1988; Bécard *et al.*, 1991; Donker and Braaksma, 1997; Peksel *et al.*, 2002; Ceccaroli *et al.*, 2003; Jobic *et al.*, 2007). In species where it has been detected, only the more intense peaks have been seen, suggesting that it is of relatively low abundance (Dijkema *et al.*, 1985; Martin *et al.*, 1998; Rangel-Castro *et al.*, 2002; Clark *et al.*, 2003).

The absence, in the labelled spectra of most replicates, of sibling peaks of arabitol, suggests that the compound was not present in large amounts, and inferred that the observed C1 peak was labelled. It has recently been shown that arabitol is accumulated in response to osmotic stress in *S. nodorum* (Lowe *et al.*, 2008). For those spectra in which labelled mannitol was abundant, the C5 peak of arabitol could not be unambiguously assigned. However, the spectra of *mpd1mdh1-107* replicates

grown on [1-^{13}C]-glucose demonstrated that both the C1 and C5 of arabitol were labelled in a 1 (C1):1.5 (C5): ratio. The fact that this strain is unique in its inability to synthesise labelled mannitol from labelled glucose has enabled this arabitol labelling pattern to be observed for the first time in fungi. Previous studies have apparently been unable to discriminate between the C1 and C5 of arabitol (Dijkema *et al.*, 1985; Martin *et al.*, 1998; Rangel-Castro *et al.*, 2002).The exception to this was the NA ^{13}C-NMR study of *Cladosporium fulvum* which did not involve a labelled substrate (Clark *et al.*, 2003). The mechanism and significance of this labelling pattern is discussed further below (Section 5.4.3.4).

5.4.2 No Third Pathway of Mannitol Metabolism Detected in *S. nodorum*

The synthesis of [1-^{13}C]-mannitol from [1-^{13}C]-glucose by both of the single mutants but not by the double mutant demonstrated that both metabolic pathways are capable of mannitol synthesis, and also that there is no alternative anabolic pathway for mannitol under these conditions.

The synthesis of [1-^{13}C]-trehalose, [6-^{13}C]-trehalose and [3-^{13}C]-alanine from [1-^{13}C=6-^{13}C]-mannitol by the *mdh1-71* strain demonstrated firstly that this strain is capable of mannitol catabolism as was concluded in Chapter 3. Secondly, it confirmed that there is an as yet unidentified component of the Mpd1 pathway by which mannitol can be phosphorylated to mannitol 1-phosphate. The inability of the *mpd1-1* mutant to catabolise mannitol indicated that at least one of the enzymatic steps catalysing the conversion of mannitol to fructose 6-phosphate by the Mdh1 pathway is under tight physiological control. Since there is no evidence of a build-up of fructose,

whilst mannitol is observed to accumulate, it is most likely that this control is applied to the step converting mannitol to fructose. It was demonstrated in the enzyme assays in Chapter 3 that the Mdh1 enzyme has the ability to oxidise mannitol in a desalted CFE. The inference is that the mannitol synthesised and/or accumulated by this strain, is in some way compartmentalised such that the Mdh1 enzyme does not have access to it, or that the catabolic reaction is subject to some form of inhibition. The inability of the *mpd1mdh1-107* mutant to catabolise mannitol indicated that there is no alternative catabolic pathway for mannitol.

5.4.3 Scrambling of Label is not Proof of a Mannitol Cycle

Label scrambling has been previously seen in a number of studies and has generally been explained by, and given as evidence of, an operational mannitol cycle (Martin *et al.*, 1988; Ramstedt *et al.*, 1989; Pfeffer and Shachar-Hill, 1996). This has not taken into account the possibility that a single pathway capable of both mannitol synthesis and catabolism would be sufficient to contribute to label scrambling. Alternative scrambling mechanisms have been suggested including the pentose phosphate pathway (in both forward and reverse flux), and the aldolase/ triosephosphate isomerase triangle (den Hollander and Shulman, 1983; Portais and Delort, 2002).

In the mannitol scrambling model [1-^{13}C]-glucose is metabolised to [1-^{13}C=6-^{13}C]-mannitol. Subsequent catabolism of mannitol, and passage to glucose 6-phosphate via gluconeogenesis, results in the synthesis of trehalose and glucose which are labelled on both the C1 and C6 carbons. The simplest version of this model would

result in equal distribution of label on the C1 and C6 of trehalose. Studies have invariably shown that this is not the case, with reported ratios of ^{13}C6 to ^{13}C1 of 0.7 (Martin *et al.*, 1985), 0.8 (Martin *et al.*, 1988) or lower (Ramstedt *et al.*, 1989). This has been explained as being due to a proportion of trehalose being synthesised directly from [1-^{13}C]-glucose, resulting in enrichment of the label in the C1 of trehalose (Peksel *et al.*, 2002). In light of the conclusion of Chapter 3 that the mannitol cycle does not exist as proposed in *S. nodorum*, this model requires some revision.

5.4.3.1 The Mdh1 Pathway does not Contribute to Label Scrambling

The first observation to consider is that the double mutant strain *mpd1mdh1-107*, when grown on [1-^{13}C]-glucose, produced trehalose which was labelled on both the C1 and C6 carbons and with a ^{13}C6/^{13}C1 ratio of 0.21 ± 0.01 (N=3). This demonstrated that label scrambling occurred in this strain. However, there was no labelling of mannitol in these samples, and when cultured on [1-^{13}C]-mannitol, there was no labelling of trehalose or any other compound. It is apparent, therefore, that scrambling is not solely due to cycling of carbon via mannitol, and that alternative scrambling pathways are operational in *S. nodorum*.

Secondly, the *mpd1-1* strain, in which the Mdh1 pathway was operational, exhibited a similar labelling pattern to the double mutant. On [1-^{13}C]-glucose it synthesised trehalose with a ^{13}C6/^{13}C1 ratio of 0.20 ± 0.00, and mannitol with a fold-labelling of 40.8 ± 4.9 above NA (N=3). On [1-^{13}C]-mannitol, there was no labelling of trehalose or any other compound. The almost identical labelling pattern of the two

mpd1 strains, and the demonstrated inability of the *mpd1-1* mutant to catabolise mannitol, indicates that the Mdh1 metabolic spur does not contribute to the scrambling observed in the *mpd1-1* strain.

Thirdly, while there was virtually no trehalose detected in the *mdh1-71* strain when grown on [1-^{13}C]-glucose, the [1-^{13}C]-mannitol grown samples all had significant amounts of labelled trehalose with a ^{13}C6/^{13}C1 ratio of 3.47 ± 1.74 (N=4). This suggests that the pathway(s) by which mannitol is metabolised in this strain, result in a trehalose scrambling pattern which is the inverse of that experimentally observed in other species, and in the *mpd1* mutants of *S. nodorum*.

The two alternative scrambling pathways proposed by den Hollander and Shulman (1983) would therefore appear to be of greater importance in the mechanism of scrambling than has previously been supposed.

5.4.3.2 The Aldose/Triosephosphate Isomerase Triangle

The aldolase/triosephosphate isomerase (TPI) triangle follows the metabolism of [1-^{13}C]-glucose to [1-^{13}C]-fructose-1,6-bisphosphate (FBP) via glycolysis (Figure 5.12). FBP aldolase reversibly cleaves FBP to [1-^{13}C]-dihydroxy acetone phosphate (DHAP) and glyceraldehyde 3-phosphate. These two intermediates of glycolysis are readily interconvertible via TPI, resulting in the production of [3-^{13}C]-glyceraldehyde 3-phosphate. Under conditions of high FBP aldolase activity, a population of FBP molecules can be generated which may in theory be labelled on the C1, C6, both terminal carbons or neither (den Hollander and Shulman, 1983). These can then be

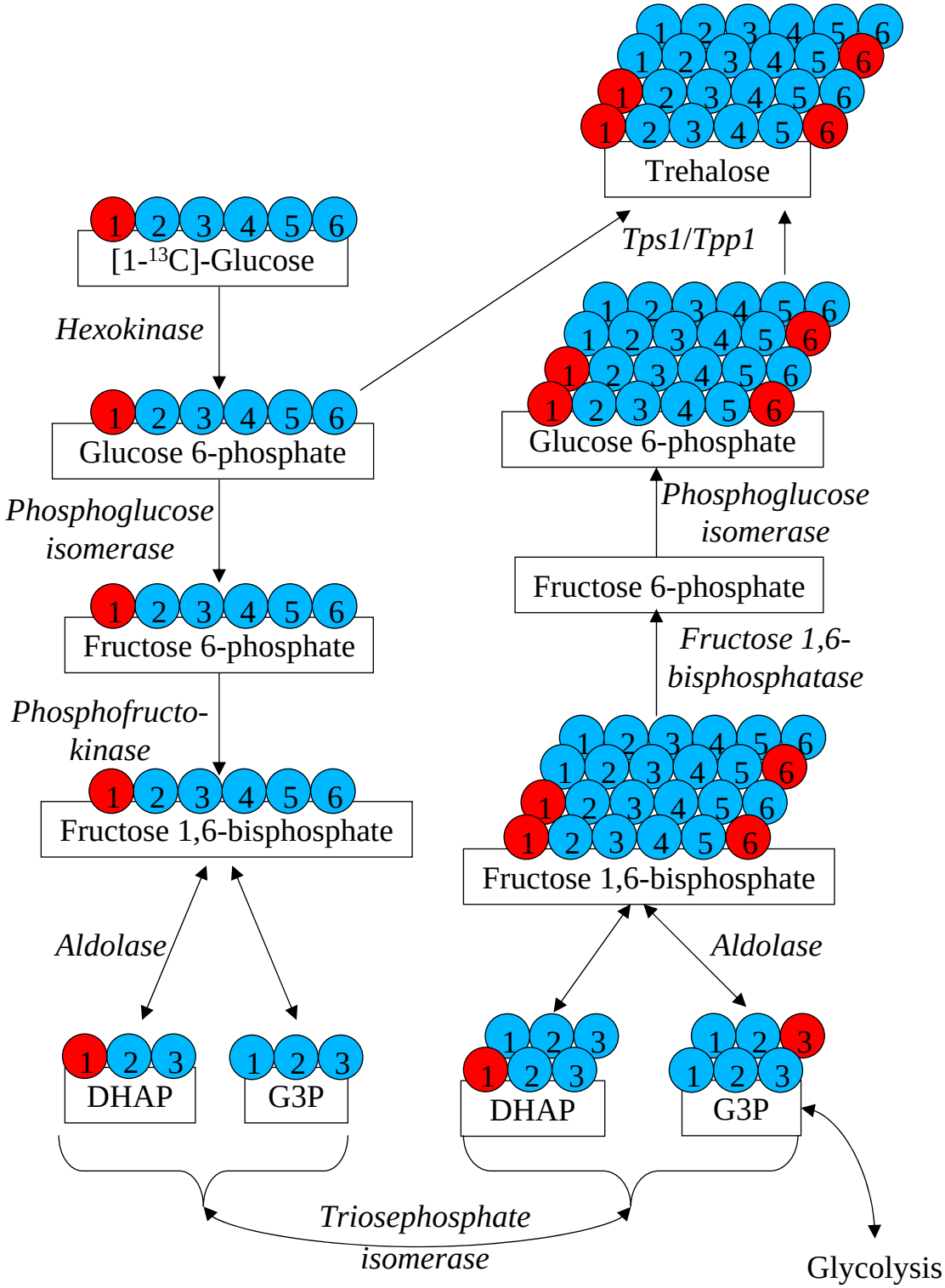


Figure 5.12: Aldolase/triosephosphate isomerase triangle mechanism for ^{13}C label scrambling from [1-^{13}C]-glucose to [1-^{13}C]/[6-^{13}C] trehalose. ^{13}C-labelled carbons are shown in red. Metabolites are shown in boxes. Enzymes are shown in italics. Abbreviations: DHAP: dihydroxy acetone phosphate; G3P: glyceraldehyde 3-phosphate*; Tps1: trehalose 6-phosphate synthase; Tpp1: trehalose 6-phosphate phosphatase.*

converted via the gluconeogenic pathway back to glucose 6-phosphate and thence to trehalose. The ^{13}C6/^{13}C1 labelling ratio of trehalose would be expected to reflect that of the glucose 6-phosphate pool from which it was formed. No glucose 6-phosphate was positively detected in any of the labelled or NA spectra. This was not surprising since intermediates of active metabolism are not often seen, and this has been attributed to their low abundance and short lifetime (Yoshida et al 1984). However, the aldolase/TPI triangle would be expected to contribute equimolar amounts of ^{13}C1/^{13}C6-labelled glucose 6-phosphate, while the passage of the labelled glucose substrate via glucose 6-phosphate would contribute to the ^{13}C1-labelled pool only. This is consistent with the trehalose labelling pattern observed for the *mpd1* mutants, but does not account for the trehalose labelling pattern of the *mdh1-71* strain grown on [1-^{13}C]-mannitol.

5.4.3.3 The Pentose Phosphate Pathway (Forward Flux)

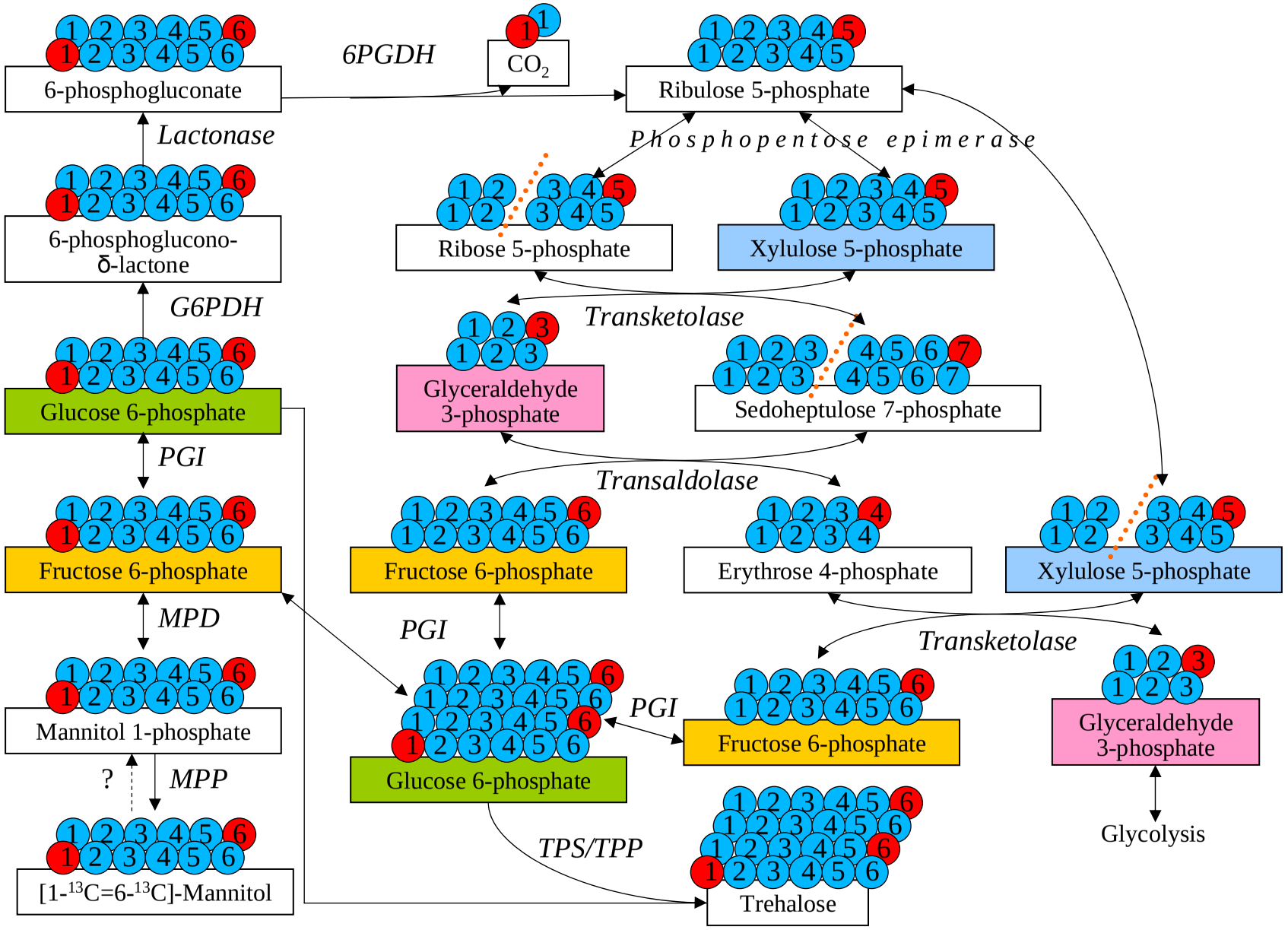
The observed formation of trehalose in the *mdh1-71* strain grown on [1-^{13}C]-mannitol, requires the conversion of mannitol to glucose 6-phosphate (G6P) via gluconeogenesis (Figure 5.13). In the absence of any other contributory pathway, the symmetry of the mannitol molecule would result in trehalose, and all intermediates, with equimolar ^{13}C1- and ^{13}C6-labelled carbon atoms. The ^{13}C6/^{13}C1 ratio of trehalose in these samples indicated that a scrambling mechanism must exist whereby labelling of the C6 is enriched to a greater extent than that of the C1. G6P can enter a number of metabolic pathways including that of trehalose biosynthesis, the pentose phosphate pathway (PPP), gluconeogenesis, glycolysis, and glycogen synthesis (Stryer, 1997). For the labelling pattern observed in trehalose to have occurred,

Figure 5.13 (overleaf): Pentose phosphate pathway mechanism for ^{13}C label scrambling from [1-^{13}C]-mannitol to [1-^{13}C]/[6-^{13}C] trehalose. ^{13}C-labelled carbons are shown in red. Metabolites are shown in boxes. Enzymes are shown in italics.

Abbreviations: MPP: mannitol 1-phosphate phosphatase; MPD: mannitol 1-phosphate dehydrogenase; PGI: phosphoglucose isomerase; G6PDH: glucose 6-phosphate dehydrogenase; 6PGDH: 6-phosphogluconate dehydrogenase; TPS: trehalose 6-phosphate synthase; TPP: trehalose 6-phosphate phosphatase.

Enzymatic cleavage sites are indicated by a dotted line. Boxes of metabolites occurring more than once are given a matching colour. Co-factors are not shown.

NB: For simplicity, the pathway from mannitol to fructose 6-phosphate via mannitol 1-phosphate only has been depicted in this figure. This was demonstrated to be the major pathway contributing to the labelling patterns observed. The alternative pathway from mannitol to fructose 6-phosphate via fructose, mediated by mannitol 2-dehydrogenase and hexokinase, could be depicted in addition to, or instead of, the depicted pathway.


6-phosphogluconate
6PGDH
CO2
Ribulose 5-phosphate
Lactonase
Phosphopentose epimerase
6-phosphoglucono-
δ-lactone
Ribose 5-phosphate
Xylulose 5-phosphate
G6PDH
Transketolase
Glyceraldehyde
3-phosphate
Sedoheptulose 7-phosphate
Glucose 6-phosphate
PGI
Transaldolase
Fructose 6-phosphate
Fructose 6-phosphate
Erythrose 4-phosphate
Xylulose 5-phosphate
MPD
PGI
Transketolase
Mannitol 1-phosphate
PGI
Fructose 6-phosphate
Glyceraldehyde
3-phosphate
Glucose 6-phosphate
?
MPP
TPS/TPP
Glycolysis
[1-13C=6-13C]-Mannitol
Trehalose

carbon must have been cycled through one or more of these alternative pathways. The PPP has previously been suggested as a means by which label scrambling could occur (den Hollander and Shulman, 1983).

During the PPP step involving the oxidation of 6-phosphogluconate, the C1 is lost to CO_2. Thus, in a population of molecules with ^{13}C-labelling of the C1 or C6, only the ^{13}C6 carbons would be retained. The action of the PPP transketolase and transaldolase enzymes would ultimately result in the synthesis of fructose 6-phosphate which was labelled on the C6 only (Figure 5.13). Conversion of this to G6P and trehalose would result in an increase in the ^{13}C6/^{13}C1 ratio. This is consistent with the observation for this strain. Action of the PPP would also yield [3-^{13}C]-glyceraldehyde 3-phosphate. This could lead to further labelling of trehalose, but only with equimolar contributions of ^{13}C6 and ^{13}C1 as outlined above (Section 5.4.3.1).

5.4.3.4 The Pentose Phosphate Pathway (Reverse Flux)

It has been previously assumed that the reactions of the non-oxidative portion of the PPP are fully reversible, and that this could contribute to label scrambling (Portais and Delort, 2002). Given a population of F6P molecules variously labelled on one, both or neither terminal carbon, this would give rise to a number of PPP intermediates reflecting this labelling (Figure 5.14). This may very well explain some of the unidentified peaks in the ^{13}C-labelled spectra acquired during this investigation, since few of these intermediates are present in the Compound Standard Library. Good evidence for this mechanism of scrambling is provided by the labelling pattern observed for arabitol in the *mpd1mdh1-107* strains. The passage of label via the

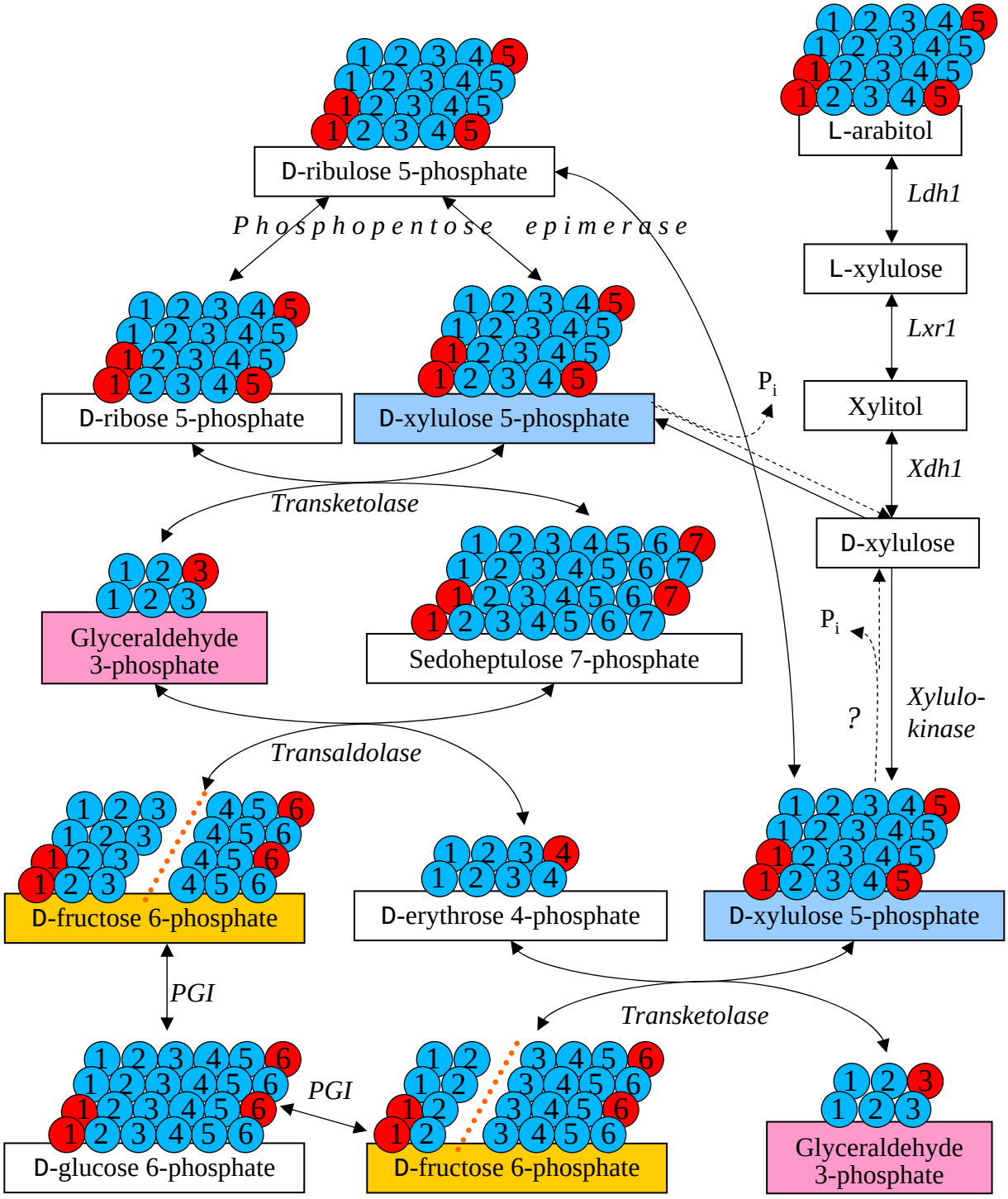


Figure 5.14: Pentose phosphate pathway mechanism for ^{13}C label scrambling from [1-^{13}C]/[6-^{13}C]-glucose 6-phosphate to [1-^{13}C]/[5-^{13}C] L-arabitol. ^{13}C-labelled carbons are shown in red. Metabolites are shown in boxes. Enzymes are shown in italics. Abbreviations: PGI: phosphoglucose isomerase; Xdh1: xylitol dehydrogenase; Lxr1: L-xylulose reductase; Ldh1: L-arabitol dehydrogenase. Enzymatic cleavage sites are indicated by a dotted line. Boxes of metabolites occurring more than once are given a matching colour. Co-factors are not shown.

reverse of the PPP is the only obvious means by which arabitol could be labelled on the C1. If the F6P pool had equal portions of label on the C1 and C6, then arabitol, *ceteris paribus*, would also have equimolar labelling of its termini. However, as seen in the spectra of the *mpd1mdh1-107* replicates grown on [1-^{13}C]-glucose, the labelling of the C5 was 1.5-fold that of the labelling on the C1. This imbalance was explained by the forward flux of the PPP which, as outlined above, lead to the preferential labelling of the C5 of arabitol.

5.4.4 Mannitol Metabolism does not Contribute to NADPH Regeneration

The main role proposed for the mannitol cycle was for the regeneration of NADPH at the expense of NADH (Hult and Gatenbeck, 1978). The evidence from the investigation of mannitol mutants in *S. nodorum* indicates that while mannitol can be synthesised and catabolised via the Mpd1 metabolic spur, and that this is the dominant pathway, only a limited amount of mannitol can be synthesised via the Mdh1 metabolic spur, and, under the experimental conditions employed in this study, this pathway is essentially irreversible. While a *de facto* cycling of mannitol would, in theory, be possible in the wild type strain, provided that both enzymes were active and had access to a common pool of mannitol, the direction of operation would be the reverse of that required for NADPH regeneration and would lead instead to net NADPH consumption. For the *mdh1-71* mutant, synthesis and catabolism of mannitol via the Mpd1 pathway effectively disassociates mannitol metabolism from NADPH regeneration. The fact that the phenotype of this mutant is unaffected by this, is evidence that NADPH regeneration is not a major role of mannitol in *S. nodorum.* Furthermore, in the *mpd1-1* mutant grown on mannitol, NADPH would be a major

product of the catabolism of mannitol via the Mdh1 pathway. However, this strain was unable to grow on mannitol as a sole carbon source. Furthermore, the enzyme assays in Chapter 3 demonstrated that the activity of the catabolic reaction was poor. It would appear, therefore, that the main pathway of NADPH regeneration is the pentose phosphate pathway.

5.4.5 Experimental Considerations

5.4.5.1 Co-located Peaks in Biological Samples Obscure Labelling

The biological extracts represented a more complex sample than the pure standards. Carbons from different compounds which had an equivalent chemical environment could not be separated on the spectrometer used. In natural abundance samples, this was apparent where a peak for a compound was more intense than it should be according to the standard spectrum. In some cases it was found that a peak from another standard compound was co-located, in other cases it was presumed that an unidentified compound was involved. In the case of the ^{13}C-labelled spectra this created a problem, since labelling might be clearly present in such a peak, but some ambiguity would arise in terms of whether the labelling was due to one or both (or more) compounds, and the percentage labelling which would be due to each. A particular case is the C5 of arabitol. In the *mpd1mdh1-107* samples grown on labelled glucose, this peak had a intensity equivalent to being labelled 12-fold above natural abundance. The ability of all other strains to metabolise glucose to mannitol, however, resulted in the presence of a mannitol C1,6 sideband which was co-located with the C5 of arabitol and prevented the determination of the amount of label in this carbon.

5.4.5.2 Low Sample Weights Affect Detection of Low Abundance Metabolites

The amount of mycelium available for analysis was quite variable between the strains. SN15 and *mdh1-71* gave consistently better yields (mean dry weight per sample of 122.7 ± 21.7 mg and 159.4 ± 30.7mg respectively) than *mpd1-1* and *mpd1mdh1-107* (66.5 ± 13.2 mg and 45.0 ± 7.0 mg respectively) when grown on 40 mM glucose. Following the extraction of polar metabolites, the mean dry weights of extracts available for analysis were 28.2 ± 8.1 mg (SN15), 21.0 ± 3.3 mg (*mdh1-71*), 12.9 ± 1.1 mg (*mpd1-1*) and 12.8 ± 3.2 mg (*mpd1mdh1-107*). Since ^{13}C-NMR is a relatively insensitive technique, low abundance metabolites were either not observed in samples with a lower dry weight, or only their most intense peaks were seen. This problem could be alleviated in future by increasing the number of cultures for the slower-growing strains and combining the harvested mycelium to bulk up the sample size.

5.4.5.3 Spectrometer Artefacts/Variation

There were a number of peaks which were observed regularly in some samples including the standards and which appeared to be artefacts. These were discounted from the analysis. It was also noted that samples which were run consecutively produced spectra which varied less from each other in terms of chemical shift, than spectra which were not consecutively run. There was some minor variation in the operating temperature of the spectrometer and this may account for some of the variation in chemical shifts between samples. The use of an internal reference compound corrected for this latter variation.

5.4.5.4 Quantification of ^{13}C Labelling

The quantification of the distribution of ^{13}C-label in NMR spectra has been approached by a number of methods. This includes comparison of the labelled peak(s) of a compound with unlabelled sibling peak(s) (Martin *et al.*, 1998; Bago *et al.*, 1999; Peksel *et al.*, 2002), and the addition of a standard of known concentration, such as EDTA (Ramstedt *et al.*, 1989; Ceccaroli *et al.*, 2003) and maleate (Jobic *et al.*, 2007). This latter approach has also been used to determine the efficiency of the extraction process (Aubert *et al.*, 1996b) and the concentration of metabolites in a spectrum.

The use of natural abundance peaks for quantification of label in labelled peaks, depends on the assumption that the natural abundance peaks themselves have not been labelled. However, the ratios of the NA peaks in the 6-carbon compounds were equivalent to those of their compound standards. This was considered sufficient evidence that there was no uniform labelling of these peaks above NA. This also constituted good evidence that NA peaks of lower-carbon-number compounds were not uniformly labelled, since cycling and scrambling of label would be revealed as perturbations in the ratios of the "NA" peaks of other compounds.

5.4.5.5 Internal Referencing of Chemical Shifts

There have been a number of internal and external standards used in fungal ^{13}C-NMR studies in order to act as a point of reference for the chemical shifts of resonance peaks. These include acetone (de Koker *et al.*, 2004), acetate (Donker and Braaksma, 1997), EDTA (Martin *et al.*, 1988; Ramstedt *et al.*, 1989; Ceccaroli *et al.*,

2003), dioxane (Yoshida *et al.*, 1984; Thomas and Baxter, 1987), hexamethyldisiloxane (Jobic *et al.*, 2007), TMS (Martin *et al.*, 1984; Martin *et al.*, 1985; Martin *et al.*, 1988; Shachar-Hill *et al.*, 1995; Martin *et al.*, 1998; Bago *et al.*, 1999) and trimethylsilyl propionate (TMSP) (Forgue *et al.*, 2006). Deficiencies have been noted with each of these and the recommendation has been made that 2,2-dimethyl-2-silapentane-5-sulfonic acid (DSS) be used as the universal standard, due to its being insensitive to variations in temperature and pH, and the fact that it is chemically inert and has a single, sharp, unambiguous highfield resonance peak (Wishart and Sykes, 1994). This recommendation was not noted until after this portion of the study had commenced. The practise of using the solvent to lock the signal, and using a compound known to be present and abundant in the samples as an internal reference, such as mannitol, glucose or trehalose, was adopted as per Dijkema *et al*. (1985). Although glycerol was the most abundant peak in some spectra, the fact that it only has two peaks, and that the more intense C1,3 peak could not always be unambiguously assigned, rendered it unsuitable as an internal reference compound. For the purposes of this study, this practise gave good correspondence between the chemical shifts of compounds in the one sample, along with the chemical shifts of their compound standards, and the same compounds in different samples. Typically, the sibling peaks of the reference compound had a variation in chemical shift of no more than 0.02 ppm from the compound standard, and the peaks of other major metabolites in the same sample had a variation of no more than 0.03 ppm. It is entirely likely that most of this variation would be explained by the compound standards having been acquired with slight variations in temperature. The use of an internal standard with the compound standards would have allowed correction for any such variation.

5.4.5.6 Limitations of Published Chemical Shifts

The identification of unknown peaks appearing in the spectra of biological samples was hindered by the lack of a more comprehensive public database of naturally occurring metabolites. While the SDBS database contains some 12,500 ^{13}C-NMR spectra (AIST, 2008), it does not at present include some compounds of interest to this study, including fructose 6-phosphate and mannitol 1-phosphate. Whilst it was a simple matter to produce standard spectra for these compounds, the identification of unknown peaks was constrained by a lack of certainty that the unknown compound was contained in the SDBS database. Furthermore, the searching function on the SDBS database does not allow for searches delimited to a particular carbon atom of a compound, but returns any carbon atom matching a submitted chemical shift parameter. When searches were conducted, they were required to take into account the observation above (Table 5.2) that the published chemical shift for a given peak could vary by as much as 2.8 ppm (after excluding the extreme chemical shifts reported in Fan (1996)), depending on the source of the data. A variation of this magnitude, even when limiting the search to 6-carbon compounds, could return well in excess of 100 possible compounds. A search function which returned records matching two or more chemical shift parameters would be of value in potential identification of unknown peaks in a spectrum.

While most published spectra agreed on the assignment of carbon atoms to particular peaks, there were a couple of exceptions to this. In the case of trehalose, there is some disagreement in the published spectra regarding the assignment of the C2 and C5 carbons. The SDBS database and some fungal ^{13}C NMR studies place the

C5 downfield of the C2 (Dijkema *et al.*, 1985; Thomas and Baxter, 1987; Bago *et al.*, 1999; Rangel-Castro *et al.*, 2002; AIST, 2008). In Fan's Chemical Tables and a smaller number of studies, the assignment of these peaks reversed (Martin *et al.*, 1985; Fan, 1996; Deveau *et al.*, 2008). While this was not of great consequence to the findings of this study in terms of the pathways by which labelling occurs, it was noted in several spectra, where the C1 of trehalose had 23-fold labelling or more above NA, that the more downfield of these two peaks exhibited spin-spin splitting. This was consistent with its being the immediate neighbouring carbon atom to the C1. It was therefore decided to adopt the less frequently applied assignment pattern in this study i.e. C2 at 72.08 ppm and C5 at 70.97 ppm.

5.4.5.7 Necessity for a Local Library of Compound Standards

Given that spectra acquired on the same instrument under the same conditions agree well with each other, it was apparent that generating a local library of compound standards conferred an advantage in the process of identifying unknown peaks. This library was designed to contain not only compounds anticipated or predicted to be involved in mannitol metabolism, but also those compounds generally involved in glycolysis, gluconeogenesis, amino acid metabolism, etc. Elimination of peaks assigned to any these compounds would better allow the identification of significantly abundant or labelled unknown peaks. In the light of the relevance of the PPP to metabolism in the strains under investigation, it would be of value to acquire standards for intermediates in this pathway.

5.4.5.8 Assumption of Labelling of Mannitol on One Terminal Carbon

It was assumed throughout the study, for the purposes of determining the labelling above NA of the C1 of mannitol, that this compound was labelled on only one terminal carbon. Since the molecule is symmetrical, it is of no consequence for the calculation whether it is the "C1" or "C6" which is labelled. Given the label-scrambling observed via the PPP and the TPI triangle, it is likely, that there would be a population of mannitol molecules produced which were labelled on both or neither of the terminal carbons. As long as these populations were of equal proportions, there should be no impact on the validity of the labelling calculation. The TPI triangle would not be expected to favour production of one population over the other. A large flux through the PPP would result in the production of unlabelled fructose 6-phosphate, but not in fructose 6-phosphate labelled on both termini. This could translate into a dilution of signal in mannitol. While this limitation of the calculation is acknowledged, it does not seem to have seriously impacted on the levels of labelling observed.

5.5 CONCLUSION

A ^{13}C-NMR investigation of carbon metabolism in the *Stagonospora nodorum* wild type strain SN15, and mutant strains with disrupted mannitol metabolism genes, revealed the operation of a number of primary metabolic pathways (Figure 5.15). It confirmed that the postulated mannitol cycle is not a necessary feature, and most likely does not exist in this species. It was demonstrated that there are two separable pathways of mannitol metabolism. The Mdh1 pathway contributed to mannitol

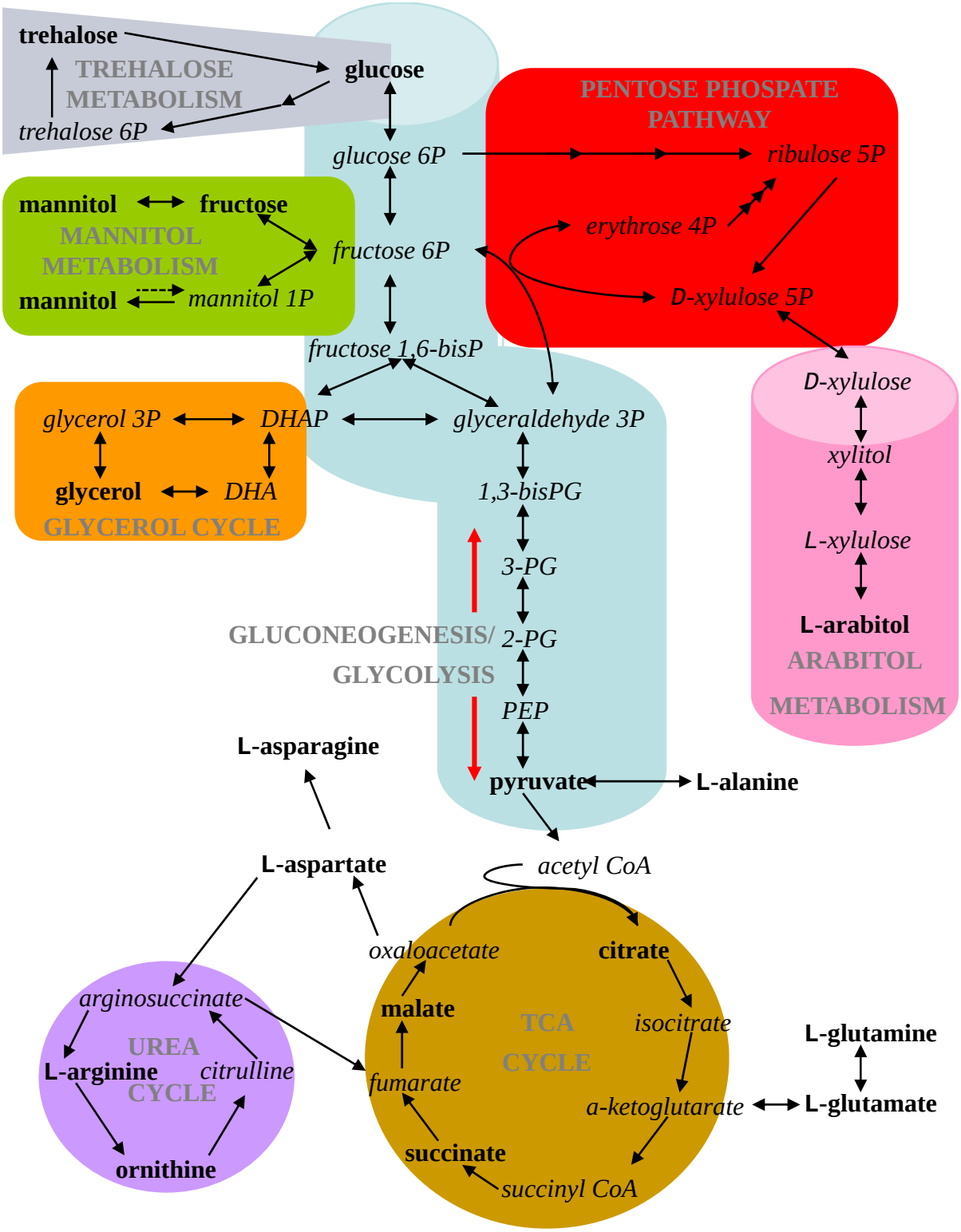


Figure 5.15: Summary of metabolic pathways demonstrated to be active in *Stagonospora nodorum*, based on the detection of metabolic intermediates in ^{13}C-NMR spectra. Detected metabolites are shown in bold and inferred metabolites in italics. Not all possible pathways for metabolism of compounds are shown. Abbreviations: P = phosphate; DHAP = dihydroxyacetone; PG = phosphoglycerate; PEP = phosphoenol pyruvate.

synthesis, but was unable to catabolise mannitol under the conditions of the experiment. The Mpd1 pathway demonstrated the capacity for mannitol synthesis and catabolism and was the dominant pathway by which this occurred. There was no third pathway of mannitol metabolism detected. It could not be determined how the Mpd1 pathway mediated the conversion of mannitol to mannitol 1-phosphate. The simplest mechanism for this conversion would require a mannitol kinase, but activity indicating the presence of such an enzyme has not been detected in this species. A number of unidentified peaks were observed in the spectra, but none which suggested the existence of an intermediate compound between mannitol and mannitol 1-phosphate.

The pattern of ^{13}C-label scrambling observed in the mutant strains was consistent with the cycling of labelled substrate via the pentose phosphate pathway and the aldose/triosephosphate isomerase triangle. Scrambling occurred in the absence of either or both of the mannitol metabolic spurs, and would not, therefore, necessarily constitute evidence of a mannitol cycle in other species. It would appear that previous studies, which have attributed the bulk of observed scrambling to a mannitol cycle, and used the degree of scrambling as an measure of the cycling between mannitol and hexose, will have resulted in an overestimate of the rapidity of this cycling (Pfeffer *et al.*, 2001).

The role proposed for mannitol of NADPH regeneration, which formed the basis of the mannitol cycle theory, is not supported by this investigation. The ^{13}C-label scrambling pattern of trehalose in the mutant strains indicated that NADPH requirements are met by the passage of carbon via the pentose phosphate pathway.

The operation of the Mpd1 pathway in isolation would make no contribution to NADPH generation, and the apparent unidirectional operation of the Mdh1 pathway in conjunction with the Mpd1 pathway would result in a *de facto* mannitol cycle operating in reverse, and resulting in NADPH consumption. There is no evidence that such a cycle was in operation.

CHAPTER 6: GENERAL CONCLUSIONS

6.1 Overview

The relationship between metabolism and pathogenicity in the wheat pathogen *Stagonospora nodorum* was investigated with specific reference to the metabolism of mannitol. This compound is one of the most abundant metabolites found in the mycelium of fungi and while a number of roles have been attributed to it, there has been little experimental evidence to support them. The metabolism of mannitol has been described as occurring in an enzymatic cycle, the principle roles of which have been for NADPH regeneration, or for dissipation of energy. It has also been suggested that mannitol has a role in phytopathogenicity. A strain of *S. nodorum* in which the mannitol 1-phosphate dehydrogenase (*Mpd1*) gene was disrupted, was unable to sporulate *in planta*, but was still able to synthesise mannitol at about 10% of wild type levels. The unidirectional nature of the theoretical mannitol cycle suggested that disruption of a mannitol 2-dehydrogenase (*Mdh1*) gene would abolish mannitol utilisation. However, a strain of *S. nodorum* in which this gene was disrupted was phenotypically identical to the wild type, retaining full pathogenicity and wild type production of mannitol. This study aimed to create a mutant harbouring both disruption constructs in order to establish the relationship between these two genes, to identify any alternative pathways of mannitol metabolism, and to attempt to abolish the metabolism of mannitol and by doing so elucidate its role in pathogenicity.

6.2 Key Findings

Disruption of both *Mdh1* and *Mpd1* genes resulted in a strain which was unable to synthesise or catabolise mannitol, although it was readily able to accumulate

mannitol from complex medium. Evidence of the inability of the strain to catabolise exogenously accumulated mannitol was provided by the lack of a demonstrated ability to grow on mannitol as a sole carbon source. Accumulated mannitol was retained as a stable pool and it required serial plating of the strain on minimal medium in which no mannitol was present, for mannitol levels to be reduced to the extent where *in vitro* sporulation was abolished. The strain was unable to sporulate *in planta*, although the addition of exogenous mannitol partially corrected this defect. This is the first time a role has been conclusively demonstrated for this compound.

A ^{13}C-NMR investigation of wild type strains and mutant strains with one or both of the *Mdh1* and *Mpd1* genes disrupted was undertaken to further characterise mannitol metabolism. Strains (including the wild type) with an intact *Mpd1* gene were characterised by having mannitol as their principal soluble carbohydrate. The *mpd1* mutants were characterised by having trehalose and glycerol as their principal soluble carbohydrates. There was no evidence to support a mannitol cycle in *S. nodorum*. It was demonstrated that cycling of labelled carbon can be explained by the triosephosphate isomerase triangle and the pentose phosphate pathway in the absence of mannitol metabolism. The importance of mannitol metabolism to carbon cycling is likely to have been overestimated in previous studies. There was no evidence to suggest that mannitol metabolism is critical to NADPH regeneration.

A metabolomics investigation of diseased versus healthy tissue from leaves inoculated with *S. nodorum* compared to mock-inoculated and uninoculated leaves did not detect any compounds which could be characterised as phytoalexins. PCA showed that the fungal specific metabolites mannitol and trehalose were associated

with diseased leaves, while the plant-specific metabolite sucrose was associated with healthy leaves.

6.3 Future Directions

It is still not apparent how the catabolism of mannitol is accomplished in the *Mpd1*-mediated pathway. There was no obvious candidate intermediate metabolite suggested by the ^{13}C-NMR investigation. If such a intermediate exists, it must be rapidly metabolised. This is not uncommon for metabolic intermediates in other pathways. While mannitol kinase activity has not previously been found in this species, the presence of such an enzyme would represent the simplest explanation for the phosphorylation of mannitol to mannitol 1-phosphate.

There were a large number of unidentified peaks observed in the ^{13}C-NMR spectra including a number which appeared to be labelled following growth upon ^{13}C-labelled substrate. These may be worth further investigation and the simplest way to progress this would be through the use of alternatively labelled substrates. The use of [2-^{13}C] or [1,2-^{13}C] would help to identify peaks belonging to the same compound. Given the flux observed through the pentose phosphate pathway, it is likely that adding intermediates of this pathway to the compound standard library would result in further identification of unknown peaks.

The fact that the abolition of synthesis of a fungal-specific metabolite leads to the inability to sporulate *in planta* is of significance for the control of this pathogen. A more complete understanding of the mechanism by which mannitol impacts on

sporulation would potentially lead to anti-fungal strategies. It would be interesting to see whether abolition of mannitol had a similar effect in other phytopathogens. It has also been demonstrated in the previous studies that the expression of mannitol catabolic genes in plants can increase their resistance to fungal pathogens. A recent study described the effects on salt and water tolerance of wheat transformed with an *E. coli* mannitol dehydrogenase gene. It would be interesting to see whether the presence of this gene conferred any improved resistance to a fungal pathogen such as *S. nodorum*.

CHAPTER 7: REFERENCES

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CHAPTER 8: APPENDICES

Table 8.1: Plant species other than *Triticum aestivum* L. subsp. *aestivum* (excluding hybrids) which have been reported as hosts of *Stagonospora nodorum*. The fungus was regarded as being pathogenic where studies have reported the plant as exhibiting disease or necrosis, or where the pathogen had caused infection/lesion formation and/or undergone pycnidiation. Instances where the literature was in conflict are noted, as are instances where the pathogen was described as causing disease, but was not reisolated from the plant. Also noted are studies where a plant was described as being a host for *S. nodorum*, but where infection was not explicitly reported.

Scientific Name	Common Name	References
Aegilops bicornis (Forsk.) Jaub. & Spach.	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops columnaris Zhuk.	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops comosa Sibth. & Smith [1]	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops crassa subsp. *crassa* Boiss.	Persian goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops cylindrica Host	jointed goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985; Khokhar and Pacumbaba, 1987)
Aegilops geniculata Roth	Ovate goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops juvenalis (Thell.) Eig.	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops kotschyi Boiss.	ovate goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops longissima Schweinf. & Muschl.	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985; Ecker *et al.*, 1990b)
Aegilops lorentii Hochst. syn. *biuncialis* Vis.	Lorent's goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops markgrafii (Greuter) K. Hammer	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops mutica Boiss.	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops neglecta subsp. *neglecta*	three-awned goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops neglecta subsp. *recta*	goatgrass	(Frauenstein and Hammer, 1985)
Aegilops peregrina (Hack.) Maire & Weiller	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)

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Table 8.1: (continued)

Scientific Name	Common Name	References
Aegilops searsii Feldman & Kislev	Sears' goatgrass	(Hammer, 1985)
Aegilops speltoides Tausch.	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985; Ecker *et al.*, 1990a)
Aegilops tauschii Cross syn. *squarrosa* L. syn. *Triticum tauschii* [2]	Tausch's goatgrass	(Jahier and Trottet, 1980; Frauenstein and Hammer, 1985; Hammer, 1985; Doussinault *et al.*, 1992; Ma and Hughes, 1993; Murphy, 1997; Murphy *et al.*, 2000; Loughman *et al.*, 2001; Murphy *et al.*, 2001)
Aegilops triuncialis L.	barbed goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops umbellulata Zhuk.	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985; Maksimov *et al.*, 2006)
Aegilops uniaristata Vis. [1]	goatgrass	(Frauenstein and Hammer, 1985; Hammer, 1985)
Aegilops ventricosa Tausch.	barbed goatgrass	(Trottet *et al.*, 1975; Frauenstein and Hammer, 1985; Hammer, 1985)
Agropyron cristatum (L.) Gaernt.	fairway crested wheatgrass	(Krupinsky, 1982; Krupinsky, 1997a)
Agropyron desertorum (Fisch. ex Link) Schultes	desert wheatgrass/ standard crested wheatgrass/clustered wheatgrass	(Krupinsky, 1982; Krupinsky, 1997a)
Agropyron fragile subsp. *Sibiricum* (Willd.) Melderis	Siberian wheatgrass	(Krupinsky, 1997a)
Agropyron repens (L.) Beauv. [3]	couchgrass/dog grass/ quackgrass	(Becker, 1957; Derevyankin, 1969; Williams and Jones, 1973; Ao and Griffiths, 1976; Rufty *et al.*, 1981b)
Agropyron spp.		(Krupinsky, 1983)
Agrostis capillaris syn. *tenuis* L.	Colonial bent	(Williams and Jones, 1973; Ao and Griffiths, 1976)
Avena fatua L. [3]	common wild oat	(Williams and Jones, 1973; Ao and Griffiths, 1976)
Avena ludoviciana Dur.	oats	(Williams and Jones, 1973; Ao and Griffiths, 1976)
Avena sativa L.	oats	(Clark and Zillinsky, 1960; Arseniuk *et al.*, 1997)
Bromus diandrus Roth. syn. *gussonii* Parl. [4]	brome grass/great brome	(Brown and Rosielle, 1980)
Bromus hordeaceus syn. *mollis* L.	soft brome	(Ao and Griffiths, 1976)
Bromus inermis Leyss. [3]	awnless brome/smooth brome/smooth bromegrass	(Dorokhova, 1967; Krupinsky, 1986b; Krupinsky, 1986a; Khokhar and Pacumbaba, 1987; Krupinsky, 1994; Krupinsky, 1997b; Krupinsky, 1997a)

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Table 8.1: (continued)

Scientific Name	Common Name	References
Bromus sterilis L.	poverty brome/barren brome/sterile brome	(Williams and Jones, 1973; Ao and Griffiths, 1976; Harrower, 1977)
Bromus tectorum L.	downy brome/early chess/ military grass/thatch bromegrass	(Fernandes, 1985)
Critesion syn. *Hordeum jubatum* L.	wild barley/foxtail barley	(Krupinsky, 1997a)
Cynodon dactylon (L.) Pers. [5]	Bermuda grass	(Khokhar and Pacumbaba, 1987)
Dactylis glomerata L. [6]	cocksfoot/orchard grass/ cocksfoot grass	(Baker, 1969)
Elymus canadensis L.	Canadian wildrye	(Krupinsky, 1997a)
Elymus histrix syn. *Hystrix patula* L.	eastern bottlebrush grass	(Rufty *et al.*, 1981b)
Elymus virginicus L.		(Rufty *et al.*, 1981b)
Elytrigia repens L.	couch grass/quackgrass	(Shearer and Zadoks, 1972; Khokhar and Pacumbaba, 1987)
Festuca elatior syn. *arundunacea* L.	tall fescue	(Ao and Griffiths, 1976; Rufty *et al.*, 1981b)
Festuca pratensis Huds.	meadow fescue	(Rufty *et al.*, 1981b)
Holcus lanatus L.	Yorkshire fog/common velvetgrass	(Williams and Jones, 1973; Ao and Griffiths, 1976)
Hordeum brachyantherum Nevski [4]	meadow barley	(Sprague, 1956)
Hordeum bulbosum L.	bulbous barley	(Rufty *et al.*, 1981b)
Hordeum hystrix Roth. [4]	barley grass/mediterranean barley grass	(Brown and Rosielle, 1980)
Hordeum marinum L.	sea barley	(Rufty *et al.*, 1981b)
Hordeum murinum L. subsp *leporinum* (Link) Arcang.	barley grass/mouse barley/ wild barley	(Brown and Rosielle, 1980)
Hordeum pusillum Nutt.	little barley	(Rufty *et al.*, 1981b; Cunfer and Youmans, 1983; Khokhar and Pacumbaba, 1987; Ueng *et al.*, 1995)

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Table 8.1: (continued)

Scientific Name	Common Name	References
Hordeum vulgare L.	barley/cereal barley/common barley	(Hansen and Magnus, 1969; Richardson and Noble, 1970; Richardson, 1972; Shearer and Zadoks, 1972; Holmes and Colhoun, 1973; Hewett, 1975; Jones, 1975; Ao and Griffiths, 1976; King, 1977; Rufty *et al.*, 1981b; Sharma *et al.*, 1982; Sharma and Brown, 1983; Cunfer, 1984; Osbourn *et al.*, 1987; Berecket *et al.*, 1990; Mansfield *et al.*, 1991; Newton and Caten, 1991; Cunfer *et al.*, 1992; Polley *et al.*, 1993; Krupinsky, 1994; Ueng *et al.*, 1995; Arseniuk *et al.*, 1997; Bousquet and Kollmann, 1998; Duczek *et al.*, 1999; Turkington *et al.*, 2002)
Hordeum vulgare L. *pallidum* Ser. [syn. *vulgare*]	barley	(Weber, 1922b)
Leymus syn. *Elymus angustus* (Trin.) Pilger	Altai wildrye	(Krupinsky, 1994; Krupinsky, 1997b; Krupinsky, 1997a)
Leymus syn. *Elymus cinereus* (Scribn. & Merr.) A. Löve	basin wildrye	(Krupinsky, 1994; Krupinsky, 1997b; Krupinsky, 1997a)
Leymus racemosis (Lam.) Tzvelev subsp. *vacemosus* [syn. *Elymus giganteus* Vahl.]	mammoth wildrye, Volga wildrye	(Krupinsky, 1997a)
Lolium multiflorum Lam.	Italian ryegrass	(Harrower, 1977; Rufty *et al.*, 1981b)
Lolium perrene L.	perennial ryegrass	(Shearer and Zadoks, 1972; Ao and Griffiths, 1976; Rufty *et al.*, 1981b; Khokhar and Pacumbaba, 1987; Jenkyn and King, 1988; Ueng *et al.*, 1995)
Lolium rigidum Gaudin [4]	annual ryegrass	(Brown and Rosielle, 1980)
Melica smithii (Porter *ex* A. Gray) Vasey[4]	Smith's melicgrass	(Sprague, 1955)
Melica subulata (Griseb.) Scribn. [4]	Alaska oniongrass	(Sprague, 1955)
Pascopyrum syn. *Agropyron smithii* (Rydb.) A. Löve	pubescent wheatgrass/ western wheatgrass	(Krupinsky, 1982; Krupinsky, 1994; Krupinsky, 1997b)
Phleum pratense L.	Timothy grass	(Ao and Griffiths, 1976; Harrower, 1977)
Poa annua L.	annual meadow-grass	(Becker, 1957; Shearer and Zadoks, 1972; Ao and Griffiths, 1976)
Poa compressa L. [7]	flattened meadow-grass	(Rufty *et al.*, 1981b)
Poa diversifolia (Boiss. & Ball.) Hack. ex Boiss.		(Rufty *et al.*, 1981b)
Poa pratensis L.	smooth meadow-grass or Kentucky bluegrass	(Weber, 1922b; Becker, 1957; Williams and Jones, 1973; Rufty *et al.*, 1981b)
Poa trivialis L.	rough bluegrass	(Williams and Jones, 1973)

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Table 8.1: (continued)

Scientific Name	Common Name	References
Psathyrostachys juncea (Fisch.) Nevski	Russian wildrye	(Krupinsky, 1997a)
Secale cereale L.	rye	(Weber, 1922b; Becker, 1957; Derevyankin, 1969; Arseniuk *et al.*, 1997; Joshi and Miedaner, 2003)
Thinopyrum syn. *Agropyron intermedium* (Host) Barkworth & Dewey	intermediate wheatgrass	(Krupinsky, 1982; Krupinsky, 1994; Krupinsky, 1997b)
Triticum aestivum L. subsp. *compactum* (Host) Mackey	club wheat/cluster wheat/ dwarf wheat/hedgehog wheat	(Weber, 1922b; Krupinsky *et al.*, 1977; Mielke, 1989)
Triticum aestivum L subsp. *macha* (Dekapr. & A. M. Menabde) Mackey		(Mielke, 1989)
Triticum aestivum L subsp. *spelta* (L.) Thell.	dinkel wheat/spelt wheat	(Weber, 1922b; Krupinsky *et al.*, 1977; Mielke, 1989; Aguilar *et al.*, 2005)
Triticum aestivum L. subsp. *sphaerococcum* (Perc.) Mackey	Indian dwarf wheat/shot wheat	(Tomerlin *et al.*, 1984)
Triticum monococcum L. subsp. *monococcum*	einkorn wheat	(Weber, 1922b; Tomerlin *et al.*, 1984; Mielke, 1989; Ma and Hughes, 1993; Singh *et al.*, 2006)
Triticum monococcum L. subsp. *aegilopoides* (Link) Thell.	wild einkorn	(Tomerlin *et al.*, 1984)
Triticum timopheevii (Zhuk.) Zhuk. subsp. *timopheevii*	Sanduri wheat	(Krupinsky *et al.*, 1977; Scharen and Eyal, 1980; Tomerlin *et al.*, 1984; Mielke, 1989; Ma and Hughes, 1993; Ma and Hughes, 1995; Singh *et al.*, 2006)
Triticum turgidum L. subsp. *carthlicum* (Nevski) A. Löve & D. Löve	Persian black wheat/Persian wheat	(Tomerlin *et al.*, 1984; Mielke, 1989)
Triticum turgidum L. subsp. *dicoccoides* (Körn. ex Ascb. & Graebn.) Thell.	wild emmer wheat	(Krupinsky *et al.*, 1977; Scharen and Eyal, 1980; Tomerlin *et al.*, 1984; Mielke, 1989; Singh *et al.*, 2006; Chu *et al.*, 2008)
Triticum turgidum L. subsp. *dicoccon* (Schrank.) Thell. syn. *T. dicoccum* Shrank.	emmer wheat, Samba wheat	(Weber, 1922b; Chona and Munjal, 1954; Krupinsky *et al.*, 1977; Tomerlin *et al.*, 1984; Mielke, 1989; Ma and Hughes, 1993; Singh *et al.*, 2006)
Triticum turgidum L. subsp. *durum* (Desf.) Husn.	durum wheat, macaroni wheat	(Weber, 1922b; Krupinsky *et al.*, 1977; Scharen and Eyal, 1980; Mullaney *et al.*, 1983; Tomerlin *et al.*, 1984; Mielke, 1989; Gilbert and Tekauz, 1992b; Ma and Hughes, 1993; Fernandez *et al.*, 1996; Cao *et al.*, 2001; Xu *et al.*, 2004a; Singh *et al.*, 2006; Singh *et al.*, 2007)
Triticum turgidum L. subsp. *polonicum* (L.) Thell.	Polish wheat	(Weber, 1922b; Mielke, 1989)
Triticum turgidum L. subsp. *turgidum*	rivet wheat/cone wheat	(Mielke, 1989)

Continued on the following page

Table 8.1: (continued)

Scientific Name	Common Name	References
Vulpia bromoides (L.) Gray [4]	squirrel tail fescue/brome fescue	(Brown and Rosielle, 1980)
NA	Triticale	(Khokhar and Pacumbaba, 1987; Valuevich *et al.*, 1992; Góral *et al.*, 1994; Ueng *et al.*, 1995; Abreu *et al.*, 1996; Arseniuk *et al.*, 1997; Arseniuk *et al.*, 1998; Oettler and Schmid, 2000; Tian *et al.*, 2005)

Notes:

[1] Reaction described by Hammer (1985) as resistant to moderately resistant

[2] Most studies report resistance due to a single locus. Ma and Hughes (1993) found *S. nodorum* caused necrosis, while Jahier and Trottet (1980) found it to be only weakly pathogenic

[3] Weber (1922b) found no disease was caused by *S. nodorum* on this species

[4] Reported as a host from which the fungus was isolated, but no disease symptoms were described

[5] Very weakly pathogenic.

[6] Slight infection only with no pycnidia produced

[7] Fungus was reported as causing infection, but was not reisolated

NA = not applicable

Table 8.2: Names (in English) which have been used to describe the disease caused by *Stagonospora nodorum* on wheat.

Disease Name	References
basal glume rot	(Salmon and Throckmorton, 1930)
dry blight (caused by three *Septoria* spp. – from the description one of them was certainly *S. nodorum*)	(Sutton, 1920)
glume blotch	(Weber, 1922a; Rosen, 1947; Doling, 1961; Scharen, 1963; Scharen and Krupinsky, 1970; Melville and Jemmett, 1971; Kees and Obst, 1972; Harrower, 1974; van der Wal and Cowan, 1974; Hampton, 1975; Kent and Strobel, 1976; Harris and Grossbard, 1978; Scharen and Bryan, 1979; Straley and Scharen, 1979; Brown and Paddick, 1980; Nelson, 1980; Allingham and Jackson, 1981; Cunfer and Johnson, 1981; Rufty *et al.*, 1981a; Babadoost and Hebert, 1982; Luke *et al.*, 1983; Karjalainen and Salovaara, 1988; Peltonen and Karjalainen, 1992; Cunfer, 1993; Aris, 1999; Halama *et al.*, 1999; Pazzagli *et al.*, 1999)
glume blotch and leaf spot	(Howard *et al.*, 1994)
Leaf and glume blotch	(Mehta, 1975; Kleijer *et al.*, 1977; Wainshilbaum and Lipps, 1991; Peltonen, 1997; Fraaije *et al.*, 2001; Agrios, 2005; Tan, 2007)
leaf spot and glume blotch	(Cunfer and Youmans, 1983; Cooley *et al.*, 1999)
leaf spot disease of wheat (in concert with *S. tritici* and *Pyrenophora tritici-repentis*)	(Scott, 1988)
leafspotting complex (in concert with *Pyrenophora tritici-repentis*)	(McFadden and Harding, 1989)
Nodorum leaf and glume blotch	(Bockus and Shroyer, 1998)
Septoria (including in concert with *Septoria tritici*)	(Tyldesley and Thompson, 1980; Rosielle and Brown, 1981)
septoria blotch	(Broscious *et al.*, 1985; Lupei *et al.*, 2000; Yusupova *et al.*, 2006)
septoria blotch disease (in concert with *Septoria tritici*)	(Hart *et al.*, 1984; Mundt *et al.*, 1995)
septoria (disease) complex (in concert with *Septoria tritici*)	(Pedersen and Hughes, 1992; Sundin *et al.*, 1999; Bockus *et al.*, 2001)
septoria glume blotch	(Rosen, 1921; Cunfer *et al.*, 1980; Scharen and Eyal, 1980; Eyal, 1981; Negassa, 1987; Ecker *et al.*, 1989; Bruno and Nelson, 1990; Bostwick *et al.*, 1993; Hu *et al.*, 1996; Tyryshkin and Ershova, 2004)
septoria leaf and glume blotch	(Watson *et al.*, 1982; Leath and Papke, 1989; Caten and Newton, 2000)
septoria leaf blotch	(Nass and Johnston, 1985; Peltonen, 1993)
septoria leaf blotch (in concert with *S. avenae* f. sp. *triticea*)	(Gilbert and Tekauz, 1992a)
septoria leaf blotch complex (in concert with *S. tritici* and *S. avenae* f. sp. *triticea*)	(Gilbert *et al.*, 1993; Sooväli *et al.*, 2006)
septoria leaf spot and glume blotch	(Shipton, 1966)

Continued on the following page

Table 8.2 (Cont.): Names (in English) which have been used to describe the disease caused by *Stagonospora nodorum* on wheat.

Disease Name	Reference
septoria nodorum blotch	(Luke *et al.*, 1985; Spadafora *et al.*, 1987; Stooksbury *et al.*, 1987; Nelson and Marshall, 1990; Scharen *et al.*, 1991; Shah and Bergstrom, 1991; Leath *et al.*, 1993; Ma and Hughes, 1993; Pedersen and Hughes, 1993; Keller *et al.*, 1994; Orth and Grybauskas, 1994; Azam Parsa and Hughes, 1995; Shah *et al.*, 1995; Dubin and Rajaram, 1996; Lemerle *et al.*, 1996; Wicki, 1997; Bhathal and Loughman, 2001; Cao *et al.*, 2001; Murphy *et al.*, 2001)
septoria nodorum leaf and glume blotch	(Oettler and Schmid, 2000)
septoria nodorum leaf blotch	(Spadafora and Cole Jr., 1987; Mergoum *et al.*, 2006)
Septoria nodorum spot	(da Luz and Bergstrom, 1986)
Septoria (caused by three *Septoria* spp. – from the description one of them was certainly *S. nodorum*)	(Sutton, 1920)
Stagonospora blotch	(Milus and Chalkley, 1997; Sundin *et al.*, 1999; De Wolf and Francl, 2000; Kim and Bockus, 2003)
Stagonospora glume blotch	(Paillard *et al.*, 2003; Schnurbusch *et al.*, 2003; Tommasini *et al.*, 2007)
Stagonospora leaf blotch	(Gaurilčikienė and Ronis, 2006)
stagonospora nodorum blotch (SNB)	(Du *et al.*, 1999; Eyal, 1999; Shah *et al.*, 2000; Cunfer *et al.*, 2001; Krupinsky and Tanaka, 2001; Mebrate and Cooke, 2001; Shah *et al.*, 2001; Czembor *et al.*, 2003; Fraser *et al.*, 2003; Arseniuk *et al.*, 2004; Feng *et al.*, 2004; Kim *et al.*, 2004; Xu *et al.*, 2004a; Aguilar *et al.*, 2005; Bennett *et al.*, 2005; Liu *et al.*, 2005; Liu *et al.*, 2006; Oliver *et al.*, 2006; Singh *et al.*, 2006; Solomon *et al.*, 2006c; Cowger and Murphy, 2007; Krupinsky *et al.*, 2007; Singh *et al.*, 2007; Ali *et al.*, 2008; Friesen *et al.*, 2008a; Friesen *et al.*, 2008b; Oliver *et al.*, 2008b; Shankar *et al.*, 2008)
Stagonospora nodorum leaf and glume blotch	(Engle *et al.*, 2006)
Stagonospora nodorum leaf blotch	(Liu *et al.*, 2004b)
wheat glume blotch	(Jordan, 1981; Makkar *et al.*, 1995; Huber *et al.*, 1996; Hou and Forman III, 2000)
wheat leaf and glume blotch	(Liu *et al.*, 2004a; Oliver *et al.*, 2008a)
wheat leaf blotch	(Kimpinski *et al.*, 1987; Kimpinski *et al.*, 1989; Troshina *et al.*, 2007)

Table 8.3: ACNFP library of ^{13}C chemical shifts, carbon assignments, peak intensities, and calculated ideal natural abundance relative peak intensities (RPI) for compound standards.

Compound and Concentration	Carbon*	Chemical Shift (ppm)	Intensity	RPI (%)
Alanine 250 mM	C1	175.70	13364.1	43.62
Alanine 250 mM	C2	50.37	23275	75.97
Alanine 250 mM	C3	16.02	30637.6	100.00
Arabinose 200 mM	βC1	96.7	7616.3	97.45
Arabinose 200 mM	αC1	92.5	3937.3	50.38
Arabinose 200 mM	-	72.38	7442.3	95.23
Arabinose 200 mM	-	71.77	7135.5	91.30
Arabinose 200 mM	-	68.63	3778	48.34
Arabinose 200 mM	-	68.52	4038.2	51.67
Arabinose 200 mM	-	68.41	7815.3	100.00
Arabinose 200 mM	-	68.36	3833.1	49.05
Arabinose 200 mM	-	66.31	6964.8	89.12
Arabinose 200 mM	-	62.38	3593.2	45.98
Arabitol 250 mM	C4	70.83	27632.1	95.41
Arabitol 250 mM	C2	70.36	27377.8	94.53
Arabitol 250 mM	C3	70.17	28961.3	100.00
Arabitol 250 mM	C1	62.95	27804.2	96.00
Arabitol 250 mM	C5	62.86	28080.6	96.96
Arginine 200 mM	C1	174.35	6600.1	69.82
Arginine 200 mM	C6	156.76	1990.1	21.05
Arginine 200 mM	C2	54.30	8795	93.04
Arginine 200 mM	C5	40.49	8133.3	86.04
Arginine 200 mM	C3	27.52	9452.5	100.00
Arginine 200 mM	C4	23.87	9143.7	96.73
Asparagine 200 mM	C4	174.39	1739.6	38.22
Asparagine 200 mM	C1	173.23	2598.2	57.08
Asparagine 200 mM	C2	51.24	4552	100.00
Asparagine 200 mM	C3	34.42	4100.8	90.09
Aspartate 37.6 mM	C4	174.24	1493.3	66.22
Aspartate 37.6 mM	C1	172.83	1172	51.97
Aspartate 37.6 mM	C2	50.62	1851.3	82.09
Aspartate 37.6 mM	C3	34.52	2255.2	100.00
Citrate 200 mM	C_6OO-	176.67	3217.8	29.62
Citrate 200 mM	$C_{1,5}$OO-	173.32	9785.3	90.09
Citrate 200 mM	βC	73.2	6467.6	59.54
Citrate 200 mM	α,γCH	43.2	10862.1	100.00
Meso-Erythritol 200 mM	C2,3	71.88	23633.4	100.00

Continued on the following page

Table 8.3: (Contd)

Compound and Concentration	Carbon*	Chemical Shift (ppm)	Intensity	RPI (%)
Meso-Erythritol 200 mM	C1,4	62.57	22642.3	95.81
Fructose 250 mM	αC2	101.41	2290.5	28.63
Fructose 250 mM	βC2	97.99	7586.6	94.83
Fructose 250 mM	αC3	80.58	2495.6	31.19
Fructose 250 mM	βC5	75.24	2356.4	29.45
Fructose 250 mM	αC5	74.33	2400.1	30.00
Fructose 250 mM	αC4	69.58	7865.1	98.31
Fructose 250 mM	βC3	69.11	8000.2	100.00
Fructose 250 mM	βC4	67.44	7339.1	91.74
Fructose 250 mM	αC1	63.77	6532.2	81.65
Fructose 250 mM	βC1	63.27	6789.6	84.87
Fructose 250 mM	βC6	62.52	1968.8	24.61
Fructose 250 mM	αC6	62.29	2292.1	28.65
Fructose 6-phosphate 100 mM	-	104.40	391.9	18.62
Fructose 6-phosphate 100 mM	-	101.44	1708.5	81.19
Fructose 6-phosphate 100 mM	-	81.75	550.8	26.18
Fructose 6-phosphate 100 mM	-	80.01	903.2	42.92
Fructose 6-phosphate 100 mM	-	79.90	1004.5	47.74
Fructose 6-phosphate 100 mM	-	75.87	357.3	16.98
Fructose 6-phosphate 100 mM	-	75.21	2104.2	100.00
Fructose 6-phosphate 100 mM	-	74.40	1787.1	84.93
Fructose 6-phosphate 100 mM	-	64.27	751.7	35.72
Fructose 6-phosphate 100 mM	-	64.21	1035	49.19
Fructose 6-phosphate 100 mM	-	62.86	405.7	19.28
Fructose 6-phosphate 100 mM	-	62.66	1767.9	84.02
Galactose 200 mM	βC1	96.39	3143.2	53.77
Galactose 200 mM	αC1	92.22	5841.5	99.92
Galactose 200 mM	βC5	75.09	3325.1	56.88
Galactose 200 mM	βC3	72.74	3001.3	51.34
Galactose 200 mM	βC2	71.81	3216	55.01
Galactose 200 mM	αC5	70.42	5846.1	100.00
Galactose 200 mM	αC4	69.25	5760.6	98.54
Galactose 200 mM	αC3	69.1	5758.2	98.50
Galactose 200 mM	βC4	68.68	3136	53.64
Galactose 200 mM	αC2	68.28	5605	95.88
Galactose 200 mM	αC6	61.13	5107.7	87.37
Galactose 200 mM	βC6	60.92	2924.6	50.03
Gluconate 200 mM	-	178.59	4887	56.04
Gluconate 200 mM	-	74.02	8705.4	99.83
Gluconate 200 mM	-	72.51	8578.5	98.38
Gluconate 200 mM	-	71.13	8692.7	99.69
Gluconate 200 mM	-	70.90	8720	100.00

Continued on the following page

Table 8.3: (Contd)

Compound and Concentration	Carbon*	Chemical Shift (ppm)	Intensity	RPI (%)
Gluconate 200 mM	-	62.56	7845.4	89.97
Glucose 200 mM	βC1	95.83	8932.9	95.71
Glucose 200 mM	αC1	92.02	5271.3	56.48
Glucose 200 mM	βC5	75.87	9187.6	98.44
Glucose 200 mM	βC3	75.68	8901.2	95.37
Glucose 200 mM	βC2	74.06	9010.7	96.54
Glucose 200 mM	αC3	72.69	5402.5	57.88
Glucose 200 mM	αC2	71.40	5799	62.13
Glucose 200 mM	αC5	71.36	5467.5	58.58
Glucose 200 mM	αC4	69.57	5723.9	61.33
Glucose 200 mM	βC4	69.52	9333.4	100.00
Glucose 200 mM	βC6	60.67	7436.7	79.68
Glucose 200 mM	αC6	60.51	4530.2	48.54
[1-^{13}C] Glucose 200 mM	βC1 sideband	108.56	326	15.23
[1-^{13}C] Glucose 200 mM	βC1 sideband	102.3	385.5	18.01
[1-^{13}C] Glucose 200 mM	βC1 sideband	96.76	487.6	22.78
[1-^{13}C] Glucose 200 mM	βC1 sideband	96.36	590.1	27.56
[1-^{13}C] Glucose 200 mM	βC1 sideband	96.13	1578.8	73.75
[1-^{13}C] Glucose 200 mM	βC1	95.83	190060.8	8878.03
[1-^{13}C] Glucose 200 mM	βC1 sideband	95.52	1201.4	56.12
[1-^{13}C] Glucose 200 mM	αC1 sideband	92.32	856.7	40.02
[1-^{13}C] Glucose 200 mM	αC1	92.02	114200.3	5334.47
[1-^{13}C] Glucose 200 mM	αC1 sideband	91.71	696.4	32.53
[1-^{13}C] Glucose 200 mM	βC5	75.87	2140.8	100.00
[1-^{13}C] Glucose 200 mM	βC3 (split)	75.71	1056.5	49.35
[1-^{13}C] Glucose 200 mM	βC3 (split)	75.65	1031.5	48.18
[1-^{13}C] Glucose 200 mM	βC2 (split)	74.35	967.5	45.19
[1-^{13}C] Glucose 200 mM	βC2 (split)	73.74	825.6	38.57
[1-^{13}C] Glucose 200 mM	αC3	72.68	1183.1	55.26
[1-^{13}C] Glucose 200 mM	αC2 (split)	71.7	558.6	26.09
[1-^{13}C] Glucose 200 mM	αC5	71.34	783.5	36.60
[1-^{13}C] Glucose 200 mM	αC2 (split)	71.08	523	24.43
[1-^{13}C] Glucose 200 mM	αC4	69.56	1366.4	63.83
[1-^{13}C] Glucose 200 mM	βC4	69.52	2091.9	97.72
[1-^{13}C] Glucose 200 mM	βC6 (split)	60.7	948.7	44.32
[1-^{13}C] Glucose 200 mM	βC6 (split)	60.64	951.5	44.45
[1-^{13}C] Glucose 200 mM	αC6 (split)	60.53	610.4	28.51
[1-^{13}C] Glucose 200 mM	αC6 (split)	60.49	602.5	28.14

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Table 8.3: (Contd)

Compound and Concentration	Carbon*	Chemical Shift (ppm)	Intensity	RPI (%)
Glucose 6-phosphate 75 mM	βC1	95.93	1773.8	84.68
Glucose 6-phosphate 75 mM	αC1	92.11	1266.9	60.48
Glucose 6-phosphate 75 mM	βC5	75.43	1781.6	85.05
Glucose 6-phosphate 75 mM	βC3	74.78	905.2	43.21
Glucose 6-phosphate 75 mM	αC2	74.68	852.1	40.68
Glucose 6-phosphate 75 mM	αC3	74.00	1840.1	87.85
Glucose 6-phosphate 75 mM	βC2	72.49	1079.5	51.53
Glucose 6-phosphate 75 mM	αC5	71.35	1141.4	54.49
Glucose 6-phosphate 75 mM	αC4	70.35	513.3	24.50
Glucose 6-phosphate 75 mM	βC4	69.05	2094.7	100.00
Glucose 6-phosphate 75 mM	βC6	63.94	754.2	36.01
Glucose 6-phosphate 75 mM	αC6	63.88	1119.1	53.43
Glutamate 200 mM	C5	181.24	5417.3	60.83
Glutamate 200 mM	C1	174.49	4948.2	55.56
Glutamate 200 mM	C2	54.53	7836	87.99
Glutamate 200 mM	C4	33.38	8888	99.80
Glutamate 200 mM	C3	26.87	8905.8	100.00
Glutamine 200 mM	C5	177.55	3507.3	32.79
Glutamine 200 mM	C1	173.9	3828.3	35.79
Glutamine 200 mM	C2	54.03	8920.7	83.41
Glutamine 200 mM	C4	30.72	8536.6	79.82
Glutamine 200 mM	C3	26.09	10695.2	100.00
Glycerol 200 mM	C2	72.01	7551.3	52.01
Glycerol 200 mM	C1,3	62.42	14519.2	100.00
Histidine 200 mM	COO(H)	172.47	4145.8	64.18
Histidine 200 mM	C_2H, ring	133.92	2808.7	43.48
Histidine 200 mM	C_5H, ring	127.31	3574.5	55.34
Histidine 200 mM	C_4H, ring	117.63	5168.2	80.01
Histidine 200 mM	αCH	53.47	6122.8	94.79
Histidine 200 mM	βCH	25.68	6459.3	100.00
Inosine 60 mM	-	158.40	1035	29.48
Inosine 60 mM	-	148.36	1396.9	39.79
Inosine 60 mM	-	146.03	2985.5	85.04
Inosine 60 mM	-	140.19	2963	84.40
Inosine 60 mM	-	124.14	1197.8	34.12
Inosine 60 mM	-	88.37	3326.8	94.76
Inosine 60 mM	-	85.52	3307.3	94.21
Inosine 60 mM	-	74.05	3510.6	100.00
Inosine 60 mM	-	70.34	3383.6	96.38
Inosine 60 mM	-	61.28	2945.1	83.89
Malate 200 mM	COO-	179.10	4947.7	51.76

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Table 8.3: (Contd)

Compound and Concentration	Carbon*	Chemical Shift (ppm)	Intensity	RPI (%)
Malate 200 mM	-	176.31	5532.3	57.87
Malate 200 mM	αCH	68.48	9559.8	100.00
Malate 200 mM	βCH	39.96	9419.1	98.53
Mannitol 1-phosphate 100 mM	M2	71.02	2810	100.00
Mannitol 1-phosphate 100 mM	M5	70.34	1438.1	51.18
Mannitol 1-phosphate 100 mM	M5	70.26	1456.6	51.84
Mannitol 1-phosphate 100 mM	M3 or M4	69.32	2804.1	99.79
Mannitol 1-phosphate 100 mM	M3 or M4	68.64	2572.4	91.54
Mannitol 1-phosphate 100 mM	M1	65.42	1150.7	40.95
Mannitol 1-phosphate 100 mM	M1	65.36	1191.5	42.40
Mannitol 1-phosphate 100 mM	M6	63.26	2550.3	90.76
Mannitol 200 mM	C2,5	70.74	20807.4	100.00
Mannitol 200 mM	C3,4	69.18	20545.4	98.74
Mannitol 200 mM	C1,6	63.16	18510.7	88.96
[1-^{13}C] Mannitol	C2 (split)	71.02	3004.2	40.06
[1-^{13}C] Mannitol	C5	70.75	7498.5	100.00
[1-^{13}C] Mannitol	C2 (split)	70.47	3481.6	46.43
[1-^{13}C] Mannitol	C3,4 (split)	69.19	7757.3	103.45
[1-^{13}C] Mannitol	C3,4 (split)	69.17	8035.6	107.16
[1-^{13}C] Mannitol	C1,6 sideband	63.69	1258.6	16.78
[1-^{13}C] Mannitol	C1,6 sideband	63.42	4656.7	62.10
[1-^{13}C] Mannitol	C1,6	63.16	634706.5	4538.71
[1-^{13}C] Mannitol	C1,6 sideband	62.87	4077.8	54.38
Mannose 200 mM	αC1	93.97	5273.5	94.40
Mannose 200 mM	βC1	93.60	2515.2	45.02
Mannose 200 mM	βC5	76.10	2674.3	47.87
Mannose 200 mM	βC3	72.98	2543.2	45.52
Mannose 200 mM	αC5	72.33	5241.6	93.83
Mannose 200 mM	βC2	71.15	2673.9	47.86
Mannose 200 mM	αC2	70.61	5233.5	93.68
Mannose 200 mM	αC3	70.16	5270.3	94.34
Mannose 200 mM	αC4	66.78	5249.5	93.97
Mannose 200 mM	βC4	66.54	2683.3	48.03
Mannose 200 mM	αC6,βC6	60.90	5586.4	100.00
Methionine 200 mM	COO(H)	174.16	6762.8	58.73
Methionine 200 mM	αCH	53.82	9733.1	84.52
Methionine 200 mM	βCH_2	29.59	10808.4	93.86
Methionine 200 mM	γCH_2	28.75	11339.4	98.47
Methionine 200 mM	S-CH_3	13.86	11515.2	100.00
Ornithine 200 mM	C1	174.05	2944.3	40.30

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Table 8.3: (Contd)

Compound and Concentration	Carbon*	Chemical Shift (ppm)	Intensity	RPI (%)
Ornithine 200 mM	C2	54.08	6298	86.21
Ornithine 200 mM	C5	38.85	5987.9	81.96
Ornithine 200 mM	C3	27.37	7305.6	100.00
Ornithine 200 mM	C4	22.71	7289.4	99.78
Phenylalanine 200 mM	COOH	173.88	2880.5	29.32
Phenylalanine 200 mM	C1, ring	135.03	3040.1	30.95
Phenylalanine 200 mM	C2,6, ring	129.31	9756.2	99.31
Phenylalanine 200 mM	C3,5, ring	129.06	9824.1	100.00
Phenylalanine 200 mM	C4, ring	127.64	4728.3	48.13
Phenylalanine 200 mM	αCH	55.98	4153.6	42.28
Phenylalanine 200 mM	βCH	36.29	4484.7	45.65
Pyruvate 200 mM	αC=O	205.09	1841.6	28.28
Pyruvate 200 mM	COO-	170.22	1407.6	21.61
Pyruvate 200 mM	βCH_3	26.43	6512.5	100.00
Serine 200 mM	C1	171.87	1038	38.19
Serine 200 mM	C3	59.67	2718.3	100.00
Serine 200 mM	C2	55.9	2133.9	78.50
Sorbitol 200 MM	C5	72.88	11689.1	99.21
Sorbitol 200 MM	C2	71.05	11781.7	100.00
Sorbitol 200 MM	C4	70.93	11691.6	99.24
Sorbitol 200 MM	C3	69.61	11612.2	98.56
Sorbitol 200 MM	C6	62.76	10577.8	89.78
Sorbitol 200 MM	C1	62.37	10662.7	90.50
Sucrose 200 mM	F2	103.57	9033.7	100.00
Sucrose 200 mM	G1	92.06	6902.1	76.40
Sucrose 200 mM	F5	81.25	6679.8	73.94
Sucrose 200 mM	F3	76.23	6707.2	74.25
Sucrose 200 mM	F4	73.85	6791.6	75.18
Sucrose 200 mM	G3	72.44	6834.6	75.66
Sucrose 200 mM	G5	72.28	7162.7	79.29
Sucrose 200 mM	G2	70.95	6958.5	77.03
Sucrose 200 mM	G4	69.08	7040.3	77.93
Sucrose 200 mM	F6	62.25	5777.4	63.95
Sucrose 200 mM	F1	61.18	5424.4	60.05
Sucrose 200 mM	G6	59.97	5541.8	61.35
Threonine 200 mM	C1	172.74	16899.4	51.42
Threonine 200 mM	C3	65.83	32865.6	100.00
Threonine 200 mM	C2	60.37	29513.6	89.80
Threonine 200 mM	C4	19.39	32329.4	98.37
Trehalose 200 mM	C1	93.16	20671	100.00
Trehalose 200 mM	C3	72.46	20285.4	98.13
Trehalose 200 mM	C2	72.09	20500.5	99.18

Continued on the following page

Table 8.3: (Contd)

Compound and Concentration	Carbon*	Chemical Shift (ppm)	Intensity	RPI (%)
Trehalose 200 mM	C5	70.98	20201.4	97.73
Trehalose 200 mM	C4	69.64	20461.4	98.99
Trehalose 200 mM	C6	60.47	16228.3	78.51
Tryptophan 200 mM	COO(H)	174.43	1699.7	68.95
Tryptophan 200 mM	C_8, ring	136.25	1154.4	46.83
Tryptophan 200 mM	C_9, ring	126.56	1277.7	51.83
Tryptophan 200 mM	C_2H, ring	124.95	2152.7	87.33
Tryptophan 200 mM	C_5H, ring	122.05	2465	100.00
Tryptophan 200 mM	C_4H, ring	119.38	2440.9	99.02
Tryptophan 200 mM	C_6H, ring	118.37	2423.1	98.30
Tryptophan 200 mM	C_7H, ring	111.86	2388.3	96.89
Tryptophan 200 mM	C_3, ring	107.39	1554.6	63.07
Tryptophan 200 mM	αCH	54.97	2212	89.74
Tryptophan 200 mM	βCH	26.3	2314.3	93.89
Xylitol 200 mM	C2,4	71.86	17475.9	100.00
Xylitol 200 mM	C3	70.72	8684.5	49.69
Xylitol 200 mM	C1,5	62.56	15821.8	90.53

* Carbon assignments given as published.